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*(Southampton)*

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# THE EFFECT OF MAMMALIAN PARATHORMONE ON THE SERUM CALCIUM LEVELS OF *FUNDULUS KANSAE* AND *FUNDULUS CATENATUS**

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(*Received* 18 *May* 1960)

**Abstract**—1. The effect of mammalian parathormone injections on the serum calcium levels of two closely related species of teleosts has been studied. It was not possible to demonstrate a direct change in serum calcium levels using flame analysis, although both the dosage and sequence of PTH injections were varied over a wide range.

2. Elevated serum calcium levels were found in female *F. kansae*, providing that PTH injections were preceded by estrogen injections.

3. During the breeding season, PTH increased $Ca^{45}$ turnover in the female *F. kansae*.

4. Male *F. kansae* gave only a slight response, of doubtful significance, to PTH injections. *F. catenatus* did not respond to the hormone.

5. It is suggested the response observed in the female *F. kansae* depends, in part, on the level of circulating estrogens.

## INTRODUCTION

DURING the past several decades, a considerable body of literature has appeared dealing with the endocrine control of calcium metabolism, especially in birds and mammals. Unfortunately, at least from the viewpoints of Comparative Endocrinology and Comparative Physiology, the mechanisms regulating calcium metabolism in heterothermic vertebrates have received little attention. Thus, although the teleosts form perhaps the largest and most diverse group among the vertebrates, parathyroid tissue was not demonstrated in this group until 1954 (Rasquin & Rosenbloom, 1954) and the first successful demonstration that mammalian parathyroid extract can effect skeletal changes in a teleost is even more recent (Budde, 1958).

Various aspects of the problem have been reviewed recently by Fontaine (1956), Bailey (1957) and Budde (1958).

The studies reported here deal with the effect of mammalian parathormone on the serum calcium levels of two closely related teleosts, and show that under certain conditions, a response to this hormone can be demonstrated.

## MATERIALS AND METHODS

*Fundulus kansae*, the plains killifish, were collected from a small stream near Petersburg, Missouri. This stream drains a salt spring, "Booneslick", and, following a rain, also has a freshwater branch which may carry a considerable flow of water

* This study was supported in part by N.S.F. Grant G-4327.

from natural run-off. Thus, these fish are often faced with rather large and rapid changes in salinity. The ability of this species to withstand very large salt gradients has been reported (Clemens & Jones, 1954).

*Fundulus catenatus*, the Ozark studfish, were collected from several Ozark streams where they are found in abundance. This species will not live for more than a few weeks at the most in slightly hypertonic sodium chloride solutions (unpublished data).

All fish were held in large concrete holding tanks for 2 weeks following capture, and were not fed. A gradual change of water was achieved by the use of overflow spouts and by allowing tap water to run into each tank continuously. Water at the University is obtained from deep wells and is not chlorinated.

Prior to use, the fish were moved to a constant temperature room held at $16{\cdot}0 \pm 0{\cdot}5$°C, and equilibrated to this temperature for 1 week. In every case, identical experimental aquaria were used. These aquaria had a surface area of 260 cm$^2$ and contained 2 l. of water. The number of fish per aquarium varied according to size, but in no case were more than 8 g of fish placed in a single aquarium.

Because most of the *F. kansae* weighed less than 1·4 g, it was necessary to develop a technique which would permit the collection of at least 25 $\mu$l of blood in order to obtain sufficient serum to carry out a calcium analysis. The following procedure proved adequate: A fish was held firmly in absorbent cloth and the heart quickly exposed. The cut tissues and pericardial fluid were blotted dry, and the ventricle punctured with a sharp scissors. A capillary tube, precoated with ammonium heparinate was inserted quickly into the ventricle. It was found that blood would flow readily into the capillary, especially if the fish was tipped downward. The sample was blown into a 0·1 ml centrifuge tube and centrifuged at 6000 *g* for 1 min. The clear serum was drawn up to the mark in a 10 $\mu$l pipet. The pipet was emptied and rinsed into 1·00 ml of 0·02% Sterox (Hartmann-Leddon Co.) which served as a diluent.

Calcium was measured by a Coleman flame photometer. Our practice has been to determine serum sodium levels, and to use the values thus obtained to prepare calcium standards containing equivalent amounts of sodium. By the repeated analysis of a number of standard serum preparations, and solutions of known composition, we are convinced that our analyses are accurate to within 3 per cent.

$Ca^{45}$ was determined in the following manner: 10 $\mu$l samples of serum were blown and rinsed into pyrex planchets. The samples were dried, ashed with fuming nitric acid, dried and counted using a thin window geiger tube. In every case, the counting error was kept to within 5 per cent.

The parathormone used was derived from beef glands (Eli Lilly and Co.). The estrogen used was estradiol benzoate (Nutritional Biochemicals Corp.) and was suspended in sesame oil. Saline (0·9%) was substituted for parathormone in the control animals.

## RESULTS

Our first attempts to demonstrate an effect of parathormone on serum calcium levels were direct experiments, i.e. parathormone in graded doses from 0·1 to 4·0 I.U. were injected and serum calcium levels measured at various time intervals

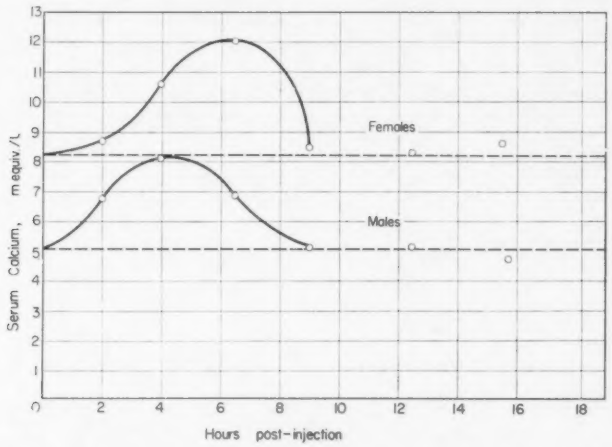


FIG. 1. Rate of calcium clearance from *F. kansae* serum. Each fish was injected intraperitoneally with 0·02 ml of 0·2 M $CaCl_2$. Each point is the average of four determinations. The broken lines represent the average serum calcium levels of the controls. The elevated serum calcium levels of the females are found during the breeding season.

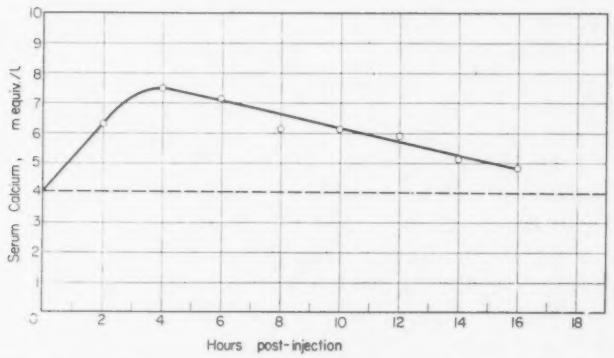


FIG. 2. Rate of calcium clearance from *F. catenatus* serum. Each fish was injected intraperitoneally with 0·02 ml of 0·3 M $CaCl_2$. Each point represents the average of four determinations. The broken line shows the average serum calcium levels of the controls. As these fish were not in breeding condition, no sex difference in serum calcium levels was found.

ranging from 2 hr to 12 days post-injection. In addition, some groups of fish were reinjected at several different time intervals and had received as much as 20 I.U. of parathormone prior to sacrifice. Further, temperature and external calcium

concentrations were varied over a wide range, but in no case were the serum calcium levels of the experimental fish significantly different from the controls.

We then attempted a variation of the parathormone assay developed by Dyer (1933) which takes advantage of the fact that this hormone accelerates the excretion rate of calcium in rats which have been maintained on a high calcium diet.

Table 1—The effect of estrogen and estrogen–PTH injections on the serum calcium levels of male and female *F. kansae*. Calcium levels are given in m equiv/l

| Controls | | Estrogen–saline | | Estrogen–PTH | |
|---|---|---|---|---|---|
| Males | Females | Males | Females | Males | Females |
| 4·0 | 7·2 | 18·7 | 17·4 | 21·6 | 23·0 |
| 4·8 | 7·4 | 9·6 | 16·1 | 11·5 | 21·4 |
| 5·9 | 6·9 | 18·1 | 16·6 | 16·6 | 22·3 |
| 5·6 | 7·3 | 16·1 | 21·6 | 18·4 | 24·0 |
| 4·8 | 7·4 | 8·5 | 18·4 | 28·8 | 31·5 |

Accordingly, calcium chloride solutions were injected intraperitoneally in graded doses until a concentration was reached which would elevate the serum calcium levels at least 50 per cent within a few hours; 0·02 ml of 0·2 M $CaCl_2$ was sufficient in the case of *F. kansae*, 0·03 ml of 0·3 M $CaCl_2$ was required in the case of *F. catenatus*. The effects of such injections on serum calcium levels are shown in Figs. 1 and 2. The fish were given a total of 8 I.U. of parathormone over a period of 1 week. Four hours after the last hormone injection, $CaCl_2$ was injected and fish were sacrificed at several different intervals ranging from 3 to 16 hr post-injection. Once again, however, the serum calcium levels of the parathormone injected fish were not, by statistical test, significantly different from the controls, although the data did suggest that parathormone was exerting some effect.

As shown in Figs. 1 and 2, calcium is cleared from the serum very rapidly, especially in the case of *F. kansae*. This suggests that, even though the fish might be responding to the hormone, there was a real possibility that any calcium mobilized might be eliminated so rapidly that it would be difficult to detect any difference from the controls, especially if only a small amount of calcium were mobilized.

In order to meet these difficulties we took advantage of some recent data reported by Bailey (1957), wherein it was shown that in the goldfish, *Carassius auratus*, estrogen injections were followed by a prompt rise in serum calcium levels. This rise was due to the presence in the serum of a protein which binds calcium firmly. The situation is apparently similar to that found in chickens (Urist, 1959). We felt that it might be possible to trap any calcium mobilized by parathormone by first injecting estrogen and thereby creating a pool of material which could trap any calcium mobilized by parathormone. The data from such an experiment are given in Table 1.

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As shown in Table 1, the serum calcium levels of female *F. kansae* which had received estrogen and parathormone injections are elevated over the control females which had received estrogen alone. The difference is significant at the 5 per cent level. Although the data are suggestive, the serum calcium levels of the males which had received estrogen and parathormone are not significantly different from

TABLE 2—THE EFFECT OF ESTROGEN AND ESTROGEN–PTH INJECTIONS ON THE SERUM CALCIUM LEVELS OF MALE AND FEMALE *F. catenatus*. CALCIUM LEVELS ARE GIVEN IN m equiv/l

| Controls | | Estrogen–saline | | Estrogen–PHT | |
|---|---|---|---|---|---|
| Males | Females | Males | Females | Males | Females |
| 4·5 | 3·3 | 11·4 | 13·0 | 11·1 | 13·7 |
| 4·3 | 4·3 | 9·4 | 11·4 | 11·6 | 11·9 |
| 4·8 | 4·1 | 10·4 | 12·7 | 9·6 | 13·5 |
| 3·7 | 4·5 | 11·0 | 11·5 | 11·5 | 12·2 |
| 4·0 | 4·0 | 12·3 | 13·9 | 11·8 | 12·6 |

TABLE 3—THE EFFECT OF PTH ON $Ca^{45}$ TURNOVER IN MALE AND FEMALE *F. kansae*. THE UNITS ARE CPM $Ca^{45}$ 10 $\mu$l SAMPLE OF SERUM. EACH NUMBER IS A DETERMINATION MADE ON A SINGLE ANIMAL

| Controls | | PTH Injected | |
|---|---|---|---|
| Males | Females | Males | Females |
| 21 | 0 | 36 | 25 |
| 26 | 0 | 37 | 56 |
| 51 | 22 | 33 | 42 |
| 26 | 20 | 49 | 36 |
| 28 | 15 | 29 | 111 |

the controls. Much to our surprise, however, *F. catenatus* gave no response whatever to parathormone injections, although they did respond to the estrogen injections. Data from an experiment using this species are given in Table 2.

In order to check these results the following experiment was carried out: Males and females of both species were weighed and matched pairs injected intraperitoneally with a 0·008 M $CaCl_2$ solution containing $Ca^{45}$. Each fish received 10 $\mu$c of isotope per gram net weight. They were then left for 1 week in order to allow the isotope time to equilibrate and exchange with non-isotopic calcium. One fish from each pair was injected with 2 I.U. of parathormone, the control received an equal volume of saline. Twelve hours later, the fish were sacrificed and the serum counted. As shown in Table 3, parathormone injections are followed by a

considerable increase in serum $Ca^{45}$ in the female *F. kansae*, and, once again, the data suggest a slight effect on the male *F. kansae*, but at no time did the confidence limits exceed 90 per cent. No differences were found in the case of *F. catenatus*.

## DISCUSSION

The data presented above show that it is possible, under the proper experimental conditions, to demonstrate a response to mammalian parathormone injections at the serum level, at least in the case of the female *F. kansae*. Since we could not show a change in serum calcium levels following parathormone treatment by flame analysis, but did find a difference in radiocalcium, it seems likely that the amount of calcium mobilized following a single injection is quite small, and that it is either excreted or reabsorbed rapidly. As yet we have not investigated the fate of the calcium mobilized, nor have we investigated its source. Since Budde (1958) has reported that short-term treatment with high doses of parathormone causes bone destruction in the guppy, *Lebistes reticulatus*, it seems not unlikely that bone is also the source of radiocalcium in this species. The data in Fig. 1 and in Table 1 suggest that calcium mobilized by parathormone is probably excreted promptly unless trapped.

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A surprising result of these investigations is that there is a clear sex difference in the response to parathormone in the case of *F. kansae*. As Budde (1958) has pointed out, all mammalian studies suggest that there is no sex difference in the response to parathormone treatment. While we cannot claim at this point that the male *F. kansae* never responds to parathormone, i.e. that the difference is absolute, we can say that the males do not respond under conditions that readily elicit a response in the females.

We are led to suggest that one factor involved in the response to parathormone is probably the level of circulating estrogens in the females. The basis for this suggestion is as follows. The radiocalcium experiments were carried out during the summer and early fall months. The females responded to parathormone readily in June, July and August, but not in September and October. These fish carry ripe eggs during the reactive period and presumably spawn a number of times during the summer months, judging from the size and number of fry present in the stream. The effect of estrogens on radiocalcium turnover during the winter months is now being investigated.

It must be pointed out, however, that while estrogen levels may be important in the case of the female *F. kansae*, other factors must also be involved. Thus, the male *F. kansae* and both sexes of *F. catenatus* show elevated serum calcium levels following estrogen injection, but do not respond to parathormone, at least under our experimental conditions. While further studies are indicated, all the data we have suggest that *F. catenatus* is completely refractory to mammalian parathormone and that the difference is one of species specificity. Such differences have been observed among mammals (Budde, 1958).

*Acknowledgements*—We are indebted to Dr. J. F. Downing of the Lilly Research Laboratories for a generous supply of parathyroid hormone.

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# COLD STRESS OF THE MAMMAL, BIRD AND REPTILE

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Department of Physiology and Biochemistry, Southampton University

(*Received* 30 *May* 1960)

**Abstract**—(1) A rise in plasma sodium and haemodilution occurs on short-term (48 hr) cold exposure (0°C) in the pigeon. These shifts are similar to those recorded for the intact rat but return more rapidly to normal. The changes are in agreement with a theory of increased mineralocorticoid activity resulting from the acute cold stress and the more rapid adaptation of the pigeon compared with the rat is in line with its greater resistance to cold.

(2) There is a haemodilution in reptiles on similar short-term cold exposure but both plasma sodium and potassium levels fall. This contrasts with the response of the rat and pigeon.

(3) In the rat on long-term (4–20 weeks) cold exposure (0°C) the plasma sodium and potassium levels are high but associated with haemodilution. These changes are accompanied by a slight fall in body temperature and pathological changes including tissue breakdown. There is a doubling of adrenal weight and histological signs of kidney and adrenal cortical damage.

(4) The pigeon on long-term (2–10 weeks) cold exposure (0°C) does not show similar changes. Plasma sodium and potassium are just below normal but again there is haemodilution. There is no fall in body temperature and the birds maintain good health with no gross pathological or histological abnormalities. The adrenal cortex shows no increase in size or weight.

(5) These results suggest that the long-term cold exposure of the intact rat may be associated with an elevated mineralocorticoid level and the cold stress produces changes similar to long-term dosage of sensitized animals with mineralocorticoids. The pigeon by contrast does not respond to a similar degree of long-term cold stress, but only shows haemodilution.

## INTRODUCTION

THE concept of stress and stress response has been widely discussed in the literature on mammalian physiology (Sayers, 1950; Selye, 1950; Selye & Heuser, 1955). A variety of stressor agents have been used in the investigations carried out on mammals and from these studies Selye developed the hypothesis that "diseases of adaptation" may be associated with a failure of adaptive mechanisms during exposure to non-specific forms of stress. Much of the evidence supporting this hypothesis has been based upon the use of previously sensitized animals, overdosed with steroid and hypophyseal hormones, by observation of the pathological and metabolic changes which follow stress (Selye & Heuser, 1955).

The evidence that exposure of sensitized animals to a stressor, under even highly artificial conditions, causes pathological changes comparable to that resulting

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from hormone administration is also limited. Thus Crane & Ingle (1958) sensitized rats by unilateral nephrectomy and a high sodium chloride diet *ad lib.* These animals were then stressed by one of the following procedures over a period of 8 weeks: surgery; limb ligation; burns; or fracture of a major bone—each once a week; haemorrhage twice a week; or injection of 1% formaldehyde twice a day. All these stressors were near the maximum which could be tolerated by the sensitized rats and yet the pathological changes in tissues of these animals were often less severe than those seen in sensitized rats overdosed with hormones, or sensitized rats with shaven hair slowly adapted to cold (3–5°C) for 60 days (Crane *et al.*, 1958).

Experiments carried out by Munday & Blane (1960) indicate that short-term cold exposure, when used as a stressor agent, in the normal intact nonsensitized rat can cause electrolyte adjustments in tissue fluids, comparable to those produced by overdosage with mineralocorticoid adrenal hormones. Such short-term cold exposure, therefore, not only provides a valuable quantitative method of analysing stress but is also of importance in that many homoiotherms in the course of their natural life come into contact with climatic conditions comparable to such varying degrees of cold stress.

The experiments described in the present paper have been carried out to determine the extent to which cold may act as a stressor in birds and reptiles. It is hoped that the results obtained from these and other experiments in progress will throw light on the evolution of homoiothermy and the related hormonic control system.

In addition the adaptive changes to long-term cold exposure of normal intact rats and pigeons have also been studied in view of the lack of direct evidence that exposure of normal animals to naturally occurring forms of stress can cause diseased conditions.

## MATERIALS AND METHODS

### *Animals*

(i) *Rats.* Rats (*Rattus norvegicus*) used in this study were adult males of the "Hooded" strain except where stated. During exposure to cold (0°C) in a large refrigerated room they were provided with their normal diet of excess rat-cake (Blue Cross No. 41B) and water *ad lib.* The rat cake was the M.R.C. Diet 41 (Parkes, 1946; Bruce & Parkes, 1949) modified by the replacement of the Cod Liver Oil by a stabilized Vitamin Supplement and known as Diet 41B.

(ii) *Pigeons.* Adult "rock-type" birds (*Columba livia*) obtained locally were maintained before and during experimentation on water and a seed mixture *ad lib.* Both before and during experiments they were kept in large well-ventilated cages, at a laboratory temperature of 21–24°C.

(iii) *Snakes.* Grass snakes (*Tropidonotus natrix*) were purchased and kept in glass tanks lined with moss and grass which was moistened every 5–7 days. They were of small to medium size and at laboratory temperatures (21–24°C) were fairly active, but did not feed.

(iv) *Lizards.* West Indian tree lizards (*Anolis gamidae*) were collected in Jamaica and used almost immediately. These animals have a normal environmental operating temperature of about 32°C and they "regulate" their body temperature by moving in and out of shade.

*Blood sampling*

For rats and pigeons blood samples were taken by cardiac puncture using a paraffined 5 ml Record Syringe and a 2 in. (No. V) Serum needle. After light anaesthesia with ether the blood samples were taken by ventricular puncture through the thoracic wall. Animals tolerated this procedure well and single samples of up to 5 ml of blood were readily obtained. Since blood sampling itself tends to cause stress, rats and pigeons were rejected after providing one sample and took no further part in any experiment.

For snakes the ether-anaesthetized animals were opened in the thoracic region and the pericardium removed. The moist ventricle was dried with filter paper and the tip snipped in diastole. The blood was then allowed to drip into a heparinized crucible.

For lizards the blood was obtained by exsanguination from an amputated limb under oral chloratose anaesthesia after intracardiac injection of heparin.

It was shown that the amount of heparin used introduced no detectable error in the plasma sodium and potassium levels.

*Procedures*

All sodium and potassium determinations were made on an "EEL" flame-photometer. Whole blood was centrifuged at 3000 rev/min for 10 min and the plasma diluted 1 : 100 in 0·05% HCl. External standards were employed containing Na, K and HCl in concentrations similar to those being determined.

Duplicate whole blood hematocrit determinations were carried out on well-mixed heparinized whole blood samples by centrifugation for 30 min at 3000 rev/min.

## RESULTS

(1) *Effect of acute (short-term) exposure to cold on plasma sodium, potassium and whole blood hematocrit*

(i) *Intact pigeons.* No published figures are available for the plasma electrolyte levels in the pigeon and consequently it was first necessary to establish the control levels. For this purpose the pigeons were maintained at a room temperature varying between 21 and 24°C and the control levels are shown in Table 1. These are similar to those reported for the chicken by Hunter (1951), i.e. Na = 146·7 m equiv/l and K = 4·06 m equiv/l.

The pigeons were exposed to cold (0 to −2°C) in several series each containing a maximum of six birds to lessen the possibility of their keeping each other warm. Blood samples were taken after 6, 12, 24, 36 and 48 hr cold exposure (Table 1).

There was a rapid rise in plasma sodium level with a highly significant maximum after 12 hr ($P < 0{\cdot}001$), but this level was not maintained and by 24 hr cold exposure the plasma sodium level was below the control level ($0{\cdot}02 < P < 0{\cdot}05$).

The 36 and 48 hr sodium levels did not vary significantly from normal.

TABLE 1—PLASMA SODIUM AND POTASSIUM CONCENTRATIONS AND WHOLE BLOOD HEMATOCRITS IN NORMAL AND COLD-EXPOSED PIGEONS.

| | Cold-exposed (hr) | Plasma electrolytes Na | K | Hematocrit (% cells) |
|---|---|---|---|---|
| Control (21–24°C) | 0 | 151·9 ± 0·45 (12) | 3·51 ± 0·22 (9) | 52·5 ± 1·07 (8) |
| Cold-exposed (0 to −2°C) | 6 | 154·5 ± 0·73 (6) | 3·57 ± 0·10 (5) | 49·7 ± 1·34 (4) |
| | 12 | 164·3 ± 1·15 (8) | 3·63 ± 0·22 (7) | 47·3 ± 1·49 (4) |
| | 24 | 149·4 ± 0·61 (6) | 3·47 ± 0·06 (5) | 42·0 ± 4·25 (5) |
| | 36 | 150·6 ± 0·77 (6) | 4·05 ± 0·32 (5) | 44·1 ± 3·20 (6) |
| | 48 | 153·7 ± 1·08 (6) | 3·04 ± 0·18 (4) | 51·9 ± 1·28 (4) |

Electrolyte concentrations in m equiv/l. Means ± standard errors. Hematocrit as percentage cells. Number of animals in parentheses.

In contrast the plasma potassium levels were much more variable at all stages and the mean change for all periods of cold exposure other than at 36 hr were statistically insignificant.

The hematocrit changes of the cold-exposed birds followed a regular pattern showing up to 20 per cent haemodilution after 24 hr with an apparently smooth

FIG. 1. Changes in whole blood hematocrit of adult pigeons on exposure to cold (0 to −2°C). Baseline of figure represents the control mean. △ net hematocrit change in % cells.

return to control levels at 48 hr (Fig. 1). This sensitivity of the blood volume in birds to stress has also been noted by Sturkie (1951). They found that the act of handling and taking a small blood sample from the breast of chickens set in motion a similar shift of water from the tissue to the blood to give haemodilution.

(ii) *Reptilia.* The body temperature of the poikilotherm follows that of the environment and therefore adjustment to cold is a normal feature of such an animal's adaptive physiological mechanism.

A comparison was made of the effect of the cold exposure on two relatively advanced poikilotherms—the grass snake (*Tropidonotus natrix*) and the West Indian tree lizard (*Anolis gamidae*).

(a) *Tropidonotus natrix.* The grass snakes became inactive soon after their tank was transferred to the cold and before the first blood samples were taken at 3 hr

TABLE 2—PLASMA SODIUM AND POTASSIUM CONCENTRATIONS AND WHOLE BLOOD HEMATOCRITS IN NORMAL AND COLD-EXPOSED GRASS SNAKES, *Tropidonotus natrix*

| | Cold-exposed (hr) | Plasma electrolytes Na | K | Hematocrit (% cells) |
|---|---|---|---|---|
| Control (21–24°C) | 0 | 159·3 (12) | 6·39 (12) | 37·0 (6) |
| Cold-exposed (0 to −2°C) | 3 | 158·8 (4) | 6·25 (4) | 31·0 (4) |
| | 6 | 159·5 (6) | 5·16 (6) | 27·5 (6) |
| | 12 | 159·8 (10) | 4·80 (4) | 27·0 (4) |
| | 24 | 159·0 (4) | 4·60 (4) | 25·5 (4) |
| | 48 | 160·7 (4) | 4·59 (4) | 21·7 (4) |

Electrolyte concentrations as means in m equiv/l. Hematocrit as percentage of cells. Number of animals in parentheses.

they appeared to have passed into a state of "hibernation". The results are summarized in Table 2 and indicate that plasma sodium remained virtually unchanged throughout the 48 hr of the experiment, but plasma potassium and the hematocrit fell fairly steadily to their lowest level at 48 hr.

(b) *Anolis gamidae.* The tree-dwelling lizards were cold-exposed in a dark refrigerator and rapidly became quite immobile, changing their body colour to dark yellow. However, on removal from the refrigerator after a 12 hr cold-exposure (8°C) period they rapidly revived and began to move about, returning to their normal green body colour.

It was not possible to determine hematocrits at the time of these experiments but the gross appearance of the blood samples after centrifugation confirmed the haemodilution shown during cold exposure in the grass snakes.

The results (Table 3) indicate that in this animal both plasma sodium and potassium levels fall after 12 hr cold exposure.

(2) *Comparison of acute exposure to cold on plasma sodium potassium and whole blood hematocrit of rats, pigeons and reptilia*

In an earlier publication (Munday & Blane, 1960), it was shown that the adrenal is implicated in the electrolyte response of the acute cold-exposed rat and

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the plasma and urine electrolyte changes are consistent with the hypothesis that cold exposure increases the endogenous production of mineralocorticoid type hormones.

TABLE 3—PLASMA SODIUM AND POTASSIUM CONCENTRATIONS IN NORMAL AND COLD-EXPOSED TREE LIZARDS, *Anolis gamidae*

| | Cold-exposed (hr) | Plasma electrolytes Na | K |
|---|---|---|---|
| Control (32°C) | 0 | 171·0 ± 5·07 (7) | 4·45 ± 0·72 (7) |
| Cold-exposed (8°C) | 12 | 151·0 ± 1·29 (6)<br>$0·01 < P < 0·02$ | 3·58 ± 0·16 (6)<br>$P < 0·10$ |

Electrolyte concentrations in m equiv/l. Means ± standard errors. Number of animals in parentheses.

TABLE 4—COMPARISON OF THE PLASMA SODIUM AND POTASSIUM CONCENTRATION CHANGES ON ACUTE EXPOSURE TO COLD OF THE INTACT RAT, PIGEON, GRASS SNAKE AND LIZARD

| Group | Control level | Time of cold exposure (% change) (hr) | | | | |
|---|---|---|---|---|---|---|
| | | 6 | 12 | 24 | 36 | 48 |
| **Sodium** | | | | | | |
| Rats | | | | | | |
| Series I | 145·6 ± 0·21 | | +8·2 | +3·5 | −0·5 | +2·2 |
| Series II | 145·6 ± 0·21 | +3·4 | +4·0 | +6·8 | +2·2 | +0·1 |
| Series III | 145·7 ± 0·56 | | +5·0 | | | |
| Pigeons | 151·9 ± 0·45 | +1·7 | +8·1 | −1·6 | −0·8 | +1·2 |
| Grass snakes | 159·3 | +0·1 | +0·3 | −0·2 | | +0·9 |
| Lizards | 171·0 ± 5·07 | | −11·6 | | | |
| **Potassium** | | | | | | |
| Rats | | | | | | |
| Series I | 4·88 ± 0·08 | | −4·5 | −12·1 | −4·5 | −0·8 |
| Series II | 4·88 ± 0·08 | +6·6 | −2·3 | −10·5 | −7·8 | −5·1 |
| Series III | 4·78 ± 0·11 | | −10·0 | | | |
| Pigeons | 3·51 ± 0·22 | +1·7 | +3·4 | −1·1 | +15·2 | −13·4 |
| Grass snakes | 6·39 | −19·4 | −25·0 | −28·2 | | −28·5 |
| Lizards | 4·45 ± 0·72 | | −19·5 | | | |

Control electrolyte concentrations in m equiv/l. Means ± standard errors. All values for exposed animals expressed as a percentage change from the control mean.

The comparative plasma electrolyte picture for the three groups is summarized in Table 4 and shows that the plasma sodium shift reported for pigeons is also consistent with such a theory. However, whereas in the mammal the initial high plasma sodium level on cold exposure takes up to 48 hr before returning to normal levels, in the pigeon it adjusts much more rapidly (i.e. before 24 hr). The plasma potassium changes in the pigeon do not follow the smooth reciprocal changes to sodium that characterize the mammal.

Both rat and pigeon show a marked haemodilution on cold exposure measured by hematocrit change. This is more marked in the bird (up to 10 per cent after 24 hr) but again, as with the plasma sodium, returns more rapidly to normal. In the mammal haemodilution is less but still increasing after 48 hr cold exposure.

By contrast the poikilotherm plasma electrolyte response to cold exposure as represented by the two reptiles studied is very different.

Both reptiles showed very considerable haemodilution; up to 58·5 per cent in the grass snake and increasing up to 48 hr cold exposure. While the plasma sodium of the grass snake remained relatively constant despite this fluid shift, the plasma potassium fell continuously though less than represented by the haemodilution effect (Table 2). In the case of the lizard both plasma sodium and potassium fell markedly.

These plasma electrolyte responses of the poikilotherm do not suggest a response to cold similar to that observed in the two homoiotherms. Also there is no evidence of any "adaptation" in the potassium and hematocrit changes over the period of this experiment with the grass snakes.



(3) *Effect of chronic (long-term) exposure to cold*

The electrolyte changes of the rat and pigeon to short-term cold exposure have been interpreted as in agreement with an hypothesis of increased mineralocorticoid release and experiments were carried out to extend this study, by comparison of the effect of long-term cold exposure on both rats and pigeons.

(i) *Rats.* Two series of rats were taken. In Series I young actively growing male hooded rats of similar body weight (49–50 g) were divided into two groups. One group of six were left in the laboratory at 21–24°C and the others placed in the cold 0 to −2°C. After 4 weeks the cold-exposed animals were in ill-health and at this time a blood sample was taken from both control and experimental animals. The animals were killed, weighed and the adrenal glands removed and weighed. Whole blood hematocrit and plasma sodium and potassium levels were determined. The results are summarized in Table 5, Series I.

The rate of growth of the cold-exposed rats is significantly depressed and the adrenal weight expressed as a percentage of total body weight more than doubled. The cold-exposed rats show a significant fall in hematocrit, and the plasma sodium and potassium levels both show a significant increase after the 4 weeks cold exposure (sodium $0{\cdot}001 < P < 0{\cdot}01$, potassium $P < 0{\cdot}001$).

The cold-exposed rats also developed extensive ulceration of the paws. The tails became oedematous and necrotic, and at the end of the 4-week period were much shortened and bleeding at their ends.

The second experimental group (Series II) consisted of thirteen Albino female rats (8 months old) of similar body weight (180–200 g). Again they were divided into two groups, one consisting of five animals—serving as controls—remaining in the laboratory at 21–24°C. The other eight were transferred to an environmental temperature of 10°C for 10 weeks, during which time they appeared to maintain

TABLE 5—BODY WEIGHT, ADRENAL WEIGHT, PLASMA SODIUM AND POTASSIUM CONCENTRATIONS AND WHOLE BLOOD HEMATOCRIT OF NORMAL AND LONG-TERM COLD-EXPOSED RATS

| | Body wt. at death (g) | Colonic temp. (°C) | Adrenal wt. (mg) | | Plasma electrolytes | | Hematocrit (% cells) |
|---|---|---|---|---|---|---|---|
| | | | Net | /100 g body wt. | Na | K | |
| Series I (Hooded rats) Control (21–24°C) | 227·0 ±11·6 (6) | | 35·7 ±1·32 (6) | 15·8 ±0·50 (6) | 145·1 ±0·32 (6) | 4·83 ±0·09 (6) | 43·3 ±0·65 (5) |
| Cold-exposed (4 weeks at 0 to −2°C) | 124·2 ±4·07 (5) | | 43·3 ±1·70 (5) | 34·9 ±0·77 (5) | 148·8 ±0·60 (5) | 5·59 ±0·16 (5) | 38·8 ±0·29 (4) |
| Series II (Albino rats) Control (21–24°C) | 237·0 ±2·24 (5) | 37·6 ±0·33 (5) | 46·3 ±1·76 (5) | 19·5 ±0·65 (5) | 145·6 ±1·15 (5) | 4·88 ±0·35 (5) | |
| Cold-exposed (10 weeks at 10°C, 11 further weeks at 0°C) | 226·3 ±4·93 (8) | 36·3 ±0·35 (8) | 70·4 ±4·03 (8) | 31·1 ±0·35 (8) | 155·9 ±2·78 (8) | 5·61 ±0·22 (7) | |

good health and showed none of the gross tissue damage reported for Series I. They were then transferred to 0°C for a further 11 weeks and at the end of this time both they and the controls were sampled as described for Series I (Table 5, Series II).

The Series II rats were older at the start of the experiment than Series I animals and were also adapted to cold exposure by a 10-week period at 10°C. By contrast with the more actively growing animals of Series I, they only just failed to maintain body weight and the body temperature was insignificantly lower than the control. Again, however, as with Series I the adrenal weight as a percentage of body weight, was significantly greater in the exposed than the control group ($P < 0{\cdot}001$).

The plasma sodium and potassium levels of the cold-exposed animals were again high as for Series I and well above control levels, but they were characterized by their wide range. Levels of 172·1 m equiv/l for sodium and 6·31 m equiv/l for potassium being recorded in the series.

At the end of the long-term cold exposure there were marked pathological changes of the gross type already reported for Series I. Tails were oedematous,

shortened and bleeding, and paws ulcerated, compared with the normal healthy appearance of either laboratory controls after 21 weeks or after the 10°C 10-week exposure period.

The adrenal glands of the cold-exposed animals showed a consistent hypertrophy of the zona fasciculata with disappearance of the demarcation between it and the zona reticularis (Fig. 2).

TABLE 6—BODY WEIGHT, ADRENAL WEIGHT, CLOACAL TEMPERATURE, PLASMA SODIUM AND POTASSIUM CONCENTRATIONS, AND WHOLE BLOOD HEMATOCRIT OF NORMAL AND LONG-TERM COLD-EXPOSED PIGEONS

| | Body wt. at death (g) | Cloacal temp. (°C) | Adrenal wt. (mg) | | Plasma electrolytes | | Hematocrit (% cells) |
|---|---|---|---|---|---|---|---|
| | | | Net | /100 g body wt. | Na | K | |
| Control (21–24°C) | 366 ±16·1 (5) | 40·5 ±0·22 (5) | 32·2 ±1·37 (5) | 8·97 ±0·73 (5) | 151·9 ±0·45(12) | 3·51 ±0·22 (9) | 52·5 ±1·07 (8) |
| Cold-exposed 0°C for 10 days | | 40·1 ±0·36 (3) | | | 147·8 ±0·98 (3) | 2·99 ±0·30 (3) | 49·0 ±1·0 (2) |
| Cold-exposed 0°C for 10 weeks | 372 ±51·1 (5) | 40·7 ±0·22 (5) | 29·2 ± 6·9 (4) | 9·34 ±3·21 (4) | 149·4 ±1·46 (5) | 2·22 ±0·21 (5) | 46·3 ±1·36 (5) |

The zona reticularis was broken up by extensive spaces which appeared to extend out towards the periphery of the gland. These changes are somewhat similar to those described by Selye (1950), Crane *et al.* (1958) and Crane & Ingle (1958) for the stressed sensitized rat.

The histology of the kidneys was more variable. While some of the cold-exposed kidneys were similar to the controls others showed signs of damage such as dilation of tubules in the medulla (Fig. 3) and breakdown and disappearance of the glomeruli in the cortex (Fig. 4) similar to that reported by Selye (1950) as a result of DCA overdosage. The pathological kidney changes were most marked in those animals with the most unhealthy gross appearance and these were also the animals in which the very high sodium and potassium plasma levels were recorded.

In contrast to certain of Selye's experiments (Selye, 1950) there was no apparent histological difference between the spleens of control and experimental animals.

(ii) *Pigeons*. Thirteen pigeons of a comparable weight and age and mixed sex were divided into three groups, one group of five were kept for a 10-week period in the laboratory at 21–24°C and used as controls.

The other group of eight birds was placed in the cold room at 0°C and three of these sampled after 10 days and the remaining five after 10 weeks. The results are summarized in Table 6 and show that the cloacal temperatures of both groups

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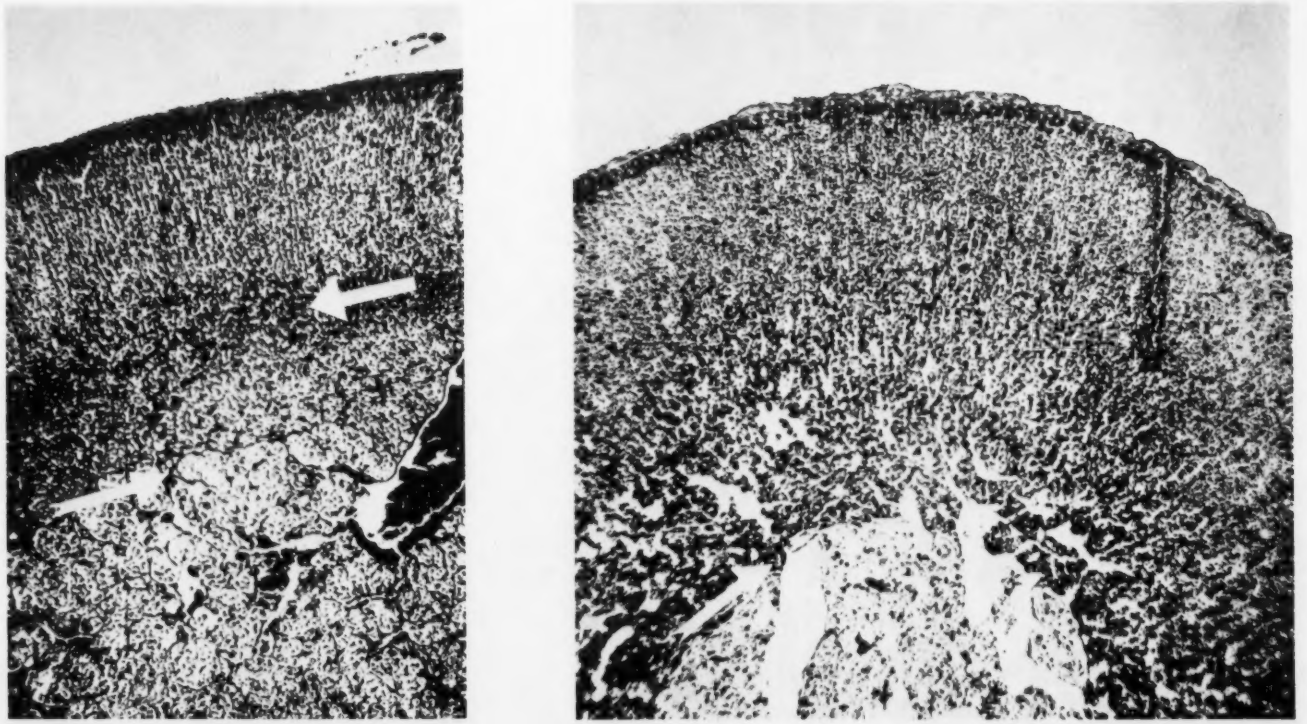
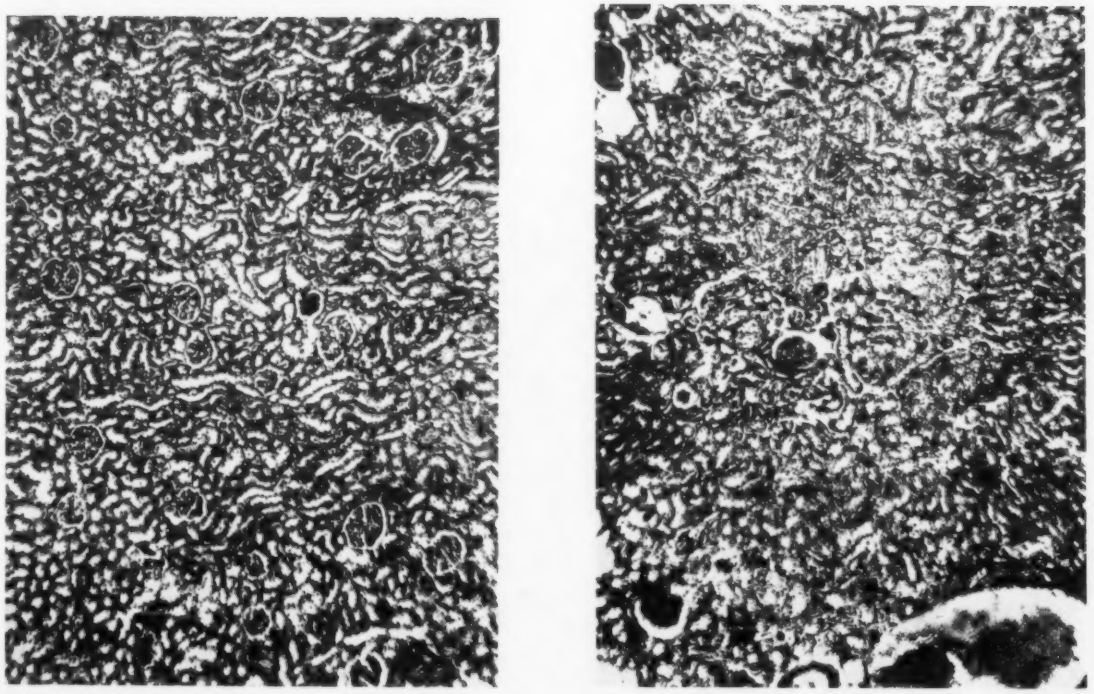


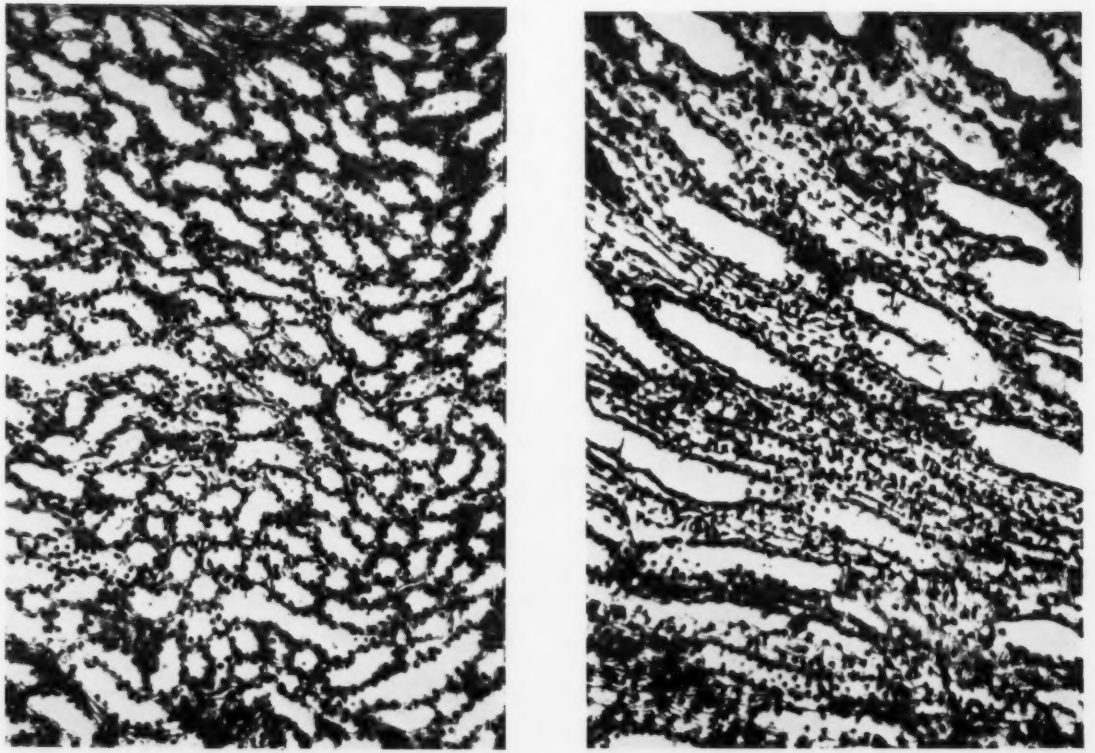
(a) (b)

FIG. 2. T.S. Rat adrenal cortex. Tissues fixed Zenker-formol. Sections cut at 8 m$\mu$. Stained in Ehrlich's hematoxylin and counterstained with eosin. Magnification $\times 62$. (a) Control—Series II: Upper arrow indicates limit of zona fasciculata, lower arrow cortex–medulla boundary. (b) Cold-exposed—Series II: 10 weeks at 10°C and 11 weeks at 0°C showing cortical hypertrophy with the formation of large spaces, and the boundary between the zona fasciculata and reticularis zones broken down.



(a) (b)

FIG. 3. T.S. Rat kidney cortex. Treated as for Fig. 2. Magnification × 62. (a) Control—Series II. (b) Cold-exposed for 10 weeks at 10°C and 11 weeks at 0°C. Series II showing relative scarcity of glomeruli, and several dark-staining atrophic glomeruli.



(a) (b)

FIG. 4. T.S. Rat kidney medulla. Treated as for Fig. 2. Magnification × 140. (a) Control—Series II. (b) Cold-exposed for 10 weeks at 10°C and 11 weeks at 0°C. Series II showing dilation of tubules.

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of cold-exposed pigeons were within the normal range. There was no sign of the elevated plasma electrolyte levels seen in the long-term cold-exposed rats. On the contrary the plasma sodium of all three 10-day exposed birds was below normal levels while the potassium was at the lower end of the normal range. In the 10-week cold-stressed birds sodium levels were mostly normal but here the potassium levels were well below the normal range. These generally lower plasma electrolyte levels may possibly correlate with the increased plasma volume indicated by the decreasing hematocrit level on cold exposure.

The control figure of 32·2 mg for adrenal weight agrees closely with the figure of 30·2 mg given for eighty-eight Carneau pigeons by Miller & Riddle (1943) but there was a wide variation in the adrenal weights of 10-week cold-exposed pigeons and consequently less significance attaches to this figure. However, it is of interest that the actual adrenal weight and the adrenal weight per 100 g body weight in these stressed birds is similar to the control levels.

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At the end of the 10-week cold exposure no gross abnormalities had developed in any of the birds in contrast to the situation in the rats. All birds appeared to be in excellent condition, and there was also no apparent histological difference between either the adrenals or the kidneys of both control and 10-week cold-exposed pigeons.

## DISCUSSION

For the short-term cold exposure the rapid and significant rise in plasma sodium and the marked haemodilution of both the rat and pigeon is in agreement with a theory of early release of mineralocorticoid in response to the stress. The more rapid return to normal of both these factors in the pigeon compared with the rat suggests a greater adaptive ability to the cold stress by the bird. This supports the suggestion in the literature that pigeons are more resistant to low temperatures (Horvath *et al.*, 1948) rather than suggesting that the pituitary–adrenal axis of birds is refractory.

While the plasma potassium shifts of the rat are also consistent with the theory of increased mineralocorticoid activity resulting from cold stress (Munday & Blane, 1960) lack of similar regular change in plasma potassium in the pigeon may have been masked by the pronounced fluid shifts, indicated by the hematocrit changes of this animal. However, it could also represent an inherent physiological difference between the hormones released by the rat and pigeon to cold stress. Plasma potassium levels are in any case closely linked with pH and carbohydrate metabolic changes and consequently the plasma sodium and fluid shifts probably provide a better index of a mineralocorticoid adrenal type of activity.

This suggestion is in line with the view that it is the extracellular sodium level which correlates most closely with fluid shifts (Robinson, 1960).

In contrast to the mammal and bird, the reptile, as a typical poikilotherm, fails to hold its body temperature even on short-term cold exposure. The absence of any rise in plasma sodium in the reptiles studied suggests that short-term cold exposure results in no excessive mineralocorticoid type of activity similar to that

observed in rats and pigeons. It might be inferred from this that cold is not acting as a stressor in the poikilotherm as it is for the homoiotherm. However, the continuously falling hematocrit and potassium level do suggest that some adaptive mechanism is in operation as a result of the cold stress.

These differing short-term cold exposure responses of the rat, pigeon and reptile draw attention to probable differences in adrenal controlling mechanisms. The limited stress experiments performed suggest that the adrenal response of birds is not necessarily mediated via the pituitary. Thus while birds did not live long without replacement therapy after hypophysectomy (Sturkie, 1954), injection of ACTH or adrenaline into intact birds resulted in no depletion of adrenal ascorbic acid although there was some adrenal hypertrophy (Jailer & Boas, 1950; Zarrow & Baldini, 1952). Young chicks were injected with agents known to produce a rapid stress response (Selye's alarm reaction) in non-adapted mammals (Bates *et al.*, 1940). Out of thyroxine, insulin, DCA, copper sulphate, potassium chloride and formaldehyde, only formaldehyde in comparatively large doses produced significant adrenal enlargement and this effect was only slightly decreased in hypophysectomized birds.

Further evidence that pituitary participation may not occur in the adrenal response to stressors in birds was provided by Miller & Riddle (1943). They found that injection of formaldehyde for 10 days was highly effective in producing adrenal hypertrophy in intact pigeons but that this same régime was even more effective in hypophysectomized birds.

In the reptiles the role of adrenocortical secretions and the relationship between the adrenal gland and the pituitary have not been closely studied although recent evidence does indicate that hormones similar to mammalian ACTH and cortisone may be produced (Chester Jones, 1957).

On long-term cold exposure of the rat and pigeon significant differences appear. While young growing rats markedly failed to maintain their body weight compared with controls, and adult rats just failed to maintain both their body weight and body temperature, pigeons maintained both body weight and temperature.

The plasma sodium of long-term cold-exposed rats was significantly higher than that of the control groups, whereas the pigeon levels were a little below the normal range.

The plasma potassium levels of long-term cold-exposed rats were significantly higher in contrast with short-term cold-exposure results. This is not in agreement with an hypothesis of increased mineralocorticoid hormone production. However, it is known that high plasma potassium levels do occur under conditions when tissue breakdown is taking place, e.g. burns. In these cases increased urinary nitrogen output parallels the potassium tissue loss (Clark & Cleghorn, 1942), and this suggests that potassium is released by the breakdown of cell walls, and the high nitrogen excretion reflects the associated protein catabolism.

There was clear evidence of tissue breakdown in all these cold-exposed rats, e.g. tail shortening, ulceration of paws, and kidney necrosis, and consequently it seems possible that the high plasma potassium levels recorded for rats might be associated

with potassium released from damaged cells. The highest levels of plasma potassium recorded in the cold-exposed rats (Table 5, Series II) were also those showing the most severe pathological tissue conditions.

The further possibility exists that the renal tubule damage reported above on long-term cold exposure in rats may have prevented normal potassium excretion.

By contrast with the rat, long-term cold exposure of the pigeon resulted in plasma potassium levels significantly below the normal range.

The decreasing hematocrit on long-term cold stress for both rats and pigeons is most probably indicative of a real increase in plasma volume. The red cell count is known to increase as a result of continued stress in mammals (Selye, 1950) and consequently a falling hematocrit is unlikely to be due to a reduced red blood-cell count. The haemodilution of short- and long-term cold exposure in the rat, pigeon and reptile contrasts with the haemoconcentration of the hypothermic mammal (Munday *et al.*, 1958). It is possible that this represents a difference between cold acting as a stressor via the adrenal in the normal homoiotherm and cold acting directly on fluid shifts in the anaesthetized homoiotherm. The haemoconcentration of the hypothermic adrenalectomized rat as compared with the haemodilution of the anaesthetized adrenalectomized rat kept at normal body temperature (Munday *et al.*, 1958) is in line with this suggestion, as is the haemodilution of the poikilotherm.

Plasma electrolyte and fluid shifts reported for the long-term cold-exposed birds are certainly not of the type that might be expected with over-production of adrenal mineralocorticoid-type hormones and in this respect pigeons are to be contrasted with rats.

The evidence from plasma sodium and fluid shifts of adrenal hyperfunction on long-term cold exposure of the rat is supported by the very significant increase in the adrenal weight ($P < 0{\cdot}001$) of both series of rats. Histological examination showed that this increase was principally associated with the enlargement of the zona fasciculata of the cortex; a region which enlarges in ACTH-treated animals and is suggested as the site of steroid synthesis (Chester Jones, 1957).

By contrast there was no significant change in the adrenal weight or histology of the long-term cold-exposed pigeon and this supports the suggestion that the bird is not responding to continued cold stress by adrenal enlargement and possible hyperfunction as did the mammal.

On the basis of experimental evidence, using mainly rats as test animals, Selye and his co-workers (Selye, 1946, 1950, 1953; Selye & Heuser, 1955) formulated and elaborated the concept of diseases of adaptation.

Although the normal response of the pituitary–adrenal system in stress is to maintain homeostasis, Selye's hypothesis holds that particularly after prolonged exposure the adrenal cortex may secrete either an excess or an imbalance of hormones and these play an aetiological role in certain diseases.

Injection of desoxycorticosterone acetate (DCA) can result in characteristic lesions of the kidney, heart and joint tissues (Selye, 1953; Ingle & Baker, 1953).

Selye (1950) has also amassed evidence that similar diseased conditions develop in stressed mammals particularly when they are sensitized, and has postulated that mineralocorticoids may be responsible.

There is, however, as yet, little available evidence of the result of naturally occurring types of stress on intact animals and also little direct evidence of increased mineralocorticoid activity in such stressed and diseased animals.

Histological examination of the adrenals and kidney of the long-term cold-exposed rats reported in this paper indicated pathological states very comparable to those induced by Selye (1950) for rats in the "exhaustion phase" of his General Adaptation Syndrome and by DCA overdosage. The plasma sodium and fluid shifts recorded for the rat have been taken as criteria of increased mineralocorticoid activity. If this is so then the pathological changes reported for the kidney and adrenal may be related to an elevated mineralocorticoid activity resulting from the cold stress.

The absence of plasma sodium shifts and adrenal enlargement of the pigeon and also the lack of gross pathological or histological changes in either the adrenal or kidney of long-term cold-exposed pigeons, upholds the earlier suggestion that pigeons are much more resistant to cold exposure than rats.

The electrolyte changes of the short-term cold-exposed pigeons would agree with a rapid mineralocorticoid-type adjustment but the long-term cold-exposed results suggest that the bird is not continuously subjected to a raised mineralocorticoid hormone level. Selye (1943) has shown that DCA dosage in young chicks for 20 days causes water retention and nephrosclerosis, and the absence of any such histological change in the kidney of these cold-exposed pigeons supports the inference that there is no continued increase in mineralocorticoid level on long-term cold exposure. It is possible that the thicker layer of insulation provided by the feathers in the pigeon and the comparatively non-vascular nature of the extremities, are factors of importance in this connexion. In addition the adaptation mechanism of birds may enable them to maintain homeostasis at low temperatures without the need for excessive pituitary–adrenal activity. Some support for this view is afforded by Zarrow & Baldini's (1952) report that exposure to cold is ineffective in depleting the adrenal ascorbic acid content of the pigeon. Such depletion occurs rapidly in the exposed rat and has been interpreted as evidence of adrenal hyperactivity.

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# TACTILE AND CHEMICAL PERCEPTION IN EARTHWORMS—II RESPONSES TO ACID pH SOLUTIONS

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**Abstract**—(1) An attempt to explain the distribution of earthworms on the basis of the sensitivity to pH is described.

(2) *A. longa* will not burrow into soil having a pH below 4·5; *L. terrestris* will not enter soil of pH 4·1 while *L. rubellus* will tolerate soils below either of these limits.

(3) Experiments involving dipping earthworms into buffer solutions confirm these observations since rejection of pH 4·4 by *A. longa*, pH 4·2 by *L. terrestris* and pH 3·8 by *L. rubellus* is very swift.

(4) Recordings from the nervous system, taken from intact or pared segmental nerves show that acid-sensitive fibres are present universally over the earthworm body surface. There is no indication that the prostomium is more sensitive than the rest of the body surface.

(5) The threshold values at which these organs respond are pH 4·6–4·4 for *A. longa*; pH 4·3–4·1 for *L. terrestris* and pH 3·8 for *L. rubellus*. They are specific to acid stimuli, not reacting to sodium chloride, quinine or sucrose.

(6) The different threshold responses reflect the different field distributions of these three species.

## INTRODUCTION

THE distribution of earthworms in nature is dependent upon a number of factors, among them the calcium content of the soil and its acidity (Jefferson, 1956; Satchell, 1956). As low calcium levels in the soil are often associated with low pH values, statistical ecological studies do not distinguish between the effects of these two parameters on earthworm population densities, and it remains uncertain whether both factors, or one only, influence earthworm distribution. It is presumed, however, that lack of calcium would exert an influence by gradual starvation (Jefferson, 1956) or possibly by altering the permeability of the body wall with a consequent inability of earthworms to control endosmosis (see discussion to Satchell's paper, 1956). Acid pH's on the other hand, may act directly upon sense organs in the earthworm body wall, producing a direct avoidance reaction and Satchell (1956) suggests that this explains the absence of certain earthworm species from highly acid soils. Other species, however, tolerate such soils and this paper attempts to explain the part played by pH in governing the distribution of various earthworm species in terms of sensory perception.

## METHODS AND MATERIALS

Three species of Lumbricidae were used for the majority of these experiments. These represent earthworms able to tolerate different pH conditions.

*Allolobophora longa* is restricted to soils of pH above 4·5. *Lumbricus terrestris* is rather more tolerant of acid conditions and has been taken at pH 4·1, whilst *L. rubellus* is "acid-tolerant" and can be found at pH 3·7. *Bimastus eisenii* and *Dendrobaena octaedra* are both small earthworms living in a restricted pH range between pH 4·8–3·7 (Satchell, 1956): their very small size unfortunately precluded their use in all but preliminary experiments.

*A. longa* and *L. terrestris* were collected from the gardens of Merlewood Research Station, Grange-over-Sands, *L. rubellus* from Roudsea Wood Nature Reserve and local sewerage tanks and *B. eisenii* and *D. octaedra* from Roudsea Wood.

Two types of gross behaviour studies were carried out. Firstly, pots of soil were taken from various sites where the soils were of different pH over a range of pH 4·0–5·0. The soil was tamped down hard so that in order to enter the soil the earthworms had to burrow actively. When this precaution was not taken it was found that worms were able to insinuate themselves into small cracks and crevices and maintained themselves in this situation by covering the burrow with secretions, thus creating an artificial habitat. Earthworms were placed on the surface of the soil, and the time taken for the animals to bury themselves was noted.

Responses to rapidly changing conditions of pH were obtained in dipping experiments. Hurwitz (1910) and Satchell (1956) suspended earthworms in solutions of various pH and determined the time taken to withdraw the first few segments from the solution. In both cases dilute acid solutions were used. In the present work Lubs–Clark buffer solutions consisting of potassium hydrogen phthalate and HCl or NaOH were used (Cole, 1955). These covered the range pH 4·9–3·2 (confirmed electrometrically).

The earthworms were suspended by the mid-region and allowed to extend into these buffer solutions. The fraction of the following minute during which the prostomium remained immersed was then noted.

Methods for recording the electrical activity of the nervous system were the same as those used previously in determining the sensitivity of *L. terrestris* and *A. longa* to other chemical and tactile stimuli (Laverack, 1960). Platinum electrodes were placed under segmental nerves and the buffer solutions applied to the external surface of the appropriate segments. Oscilloscope recordings were made of the nervous activity resulting from such stimulation. (For fuller details of the method see Laverack, 1960.) The preparation was washed between each application of test solution with distilled water, which was then removed by filter paper wicks. Distilled water had no separate effect, serving only to remove the test solution and enabling the nervous activity to return to its original level. It does not provoke impulses in the segmental nerves.

## RESULTS

### *Pot experiments*

When specimens of *A. longa* were placed on the surface of soils of pH 4·9–4·6 they were found to burrow into the soil and buried themselves within a few hours.

Earthworms of this species placed on soils of pH 4·4–4·0, however, became very active and performed a considerable amount of searching, lifting the prostomium and dabbing down with it at various places, but at no time showing signs of burrowing. The animals appeared to be carrying out a "tasting" of the ground. After 24 hr the same pattern of behaviour was shown, but after 48 hr the earthworms were inactive on a small area of soil. This region had been covered with exudate from the animals; it had dried and formed a film which presumably isolated the earthworms from contact with the soil. Subsequently the animals became desiccated and died.

*L. terrestris* from Merlewood garden (pH 5·8) would not burrow into soils of pH 4·1 even though a very few individuals of this species have been collected from soils of this acidity in the Roudsea Wood Nature Reserve. It may be that certain individuals are able to tolerate rather more acid conditions than the bulk of the population. This species will burrow into all soils of pH 4·4 and above within a short period of time.

*Bimastus eiseni* and *Dendrobaena octaedra*, two acid tolerant species, are normally found in the litter layers on top of the soil, but under the conditions described for *A. longa* they will burrow into soils of pH 4·1–4·3 within a few hours. They will also burrow into less acid garden soils (pH 4·4–5·0). *L. rubellus*, also an acid-tolerant species, burrowed into all the soils tested which ranged from pH 4·0–4·9.

Satchell (1956) found that *A. chlorotica* would burrow into soils of pH 5·0 and above, but not into those of pH 4·4 and below.

*Dipping experiments*

*A. longa*, *L. terrestris* and *L. rubellus* were gently held in the middle of the body and as they extended were allowed to submerge the prostomium and the first ten to fifteen segments into Lubs–Clark buffer solutions. The time taken for withdrawal of the anterior end was recorded and the prostomium was then rinsed with distilled water before being dipped into the next buffer.

*A. longa* extended into phthalate buffers of pH 4·9, 4·7 and 4·5. The animals remained extended and generally swept through the solutions searching in all directions, making no attempt to withdraw. In pH 4·5 this activity seemed more vigorous. When dipped into pH 4·4, however, the animals twisted rapidly, and soon contracted and removed the prostomium from the buffer. This response became even more vigorous at pH 4·3 and the prostomium barely cut the surface of the water before it was withdrawn. Table 1 shows that there is a certain amount of variation between individual worms in their response to pH and this is indicated particularly by the threshold level of pH required to induce rapid withdrawal from the buffer. Thus four out of five individuals had threshold values at pH 4·4 and the last at 4·5. At pH's less than 4·4 all the earthworms withdrew at similar speed. Fig. 1 plots the average withdrawal time obtained for each level of pH but the threshold differences between individuals should be borne in mind.

*L. terrestris*. The behaviour of *L. terrestris* was somewhat different. Animals dipped into solutions of pH 4·9–4·4 were apparently unaffected by such treatment.

TABLE 1

| pH | *A. longa* | | | | | | *L. terrestris* | | | | | | *L. rubellus* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | Av. | 1 | 2 | 3 | 4 | 5 | Av. | 1 | 2 | 3 | 4 | 5 | Av. |
| Aq. dist | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 4·9 | * | * | * | * | * | * | | | | | | | | | | | | |
| 4·8 | | | | | | | | | | | | | | | | | | |
| 4·7 | * | * | 52 | * | * | 58 | * | * | * | * | * | * | | | | | | |
| 4·6 | | | | | | | | | | | | | | | | | | |
| 4·5 | * | * | 7 | * | * | 49 | * | * | * | * | * | * | | | | | | |
| 4·4 | 11 | 13 | 8 | 11 | 7 | 10 | * | * | * | * | * | * | * | * | * | * | * | * |
| 4·3 | 11 | 10 | 7 | 5 | 14 | 9·4 | 13 | * | 37 | * | 23 | 38 | * | 45 | * | * | * | 53 |
| 4·2 | 5 | 9·5 | 3 | 4 | 1·5 | 4·6 | 9·5 | 52 | 40 | * | 22 | 37 | 55 | 45 | * | 40 | * | 52·5 |
| 4·1 | | | | | | | | | | | | | * | 35 | * | 55 | 55 | 53 |
| 4·0 | 3 | 1·5 | 1 | 1·5 | 2 | 1·6 | 23 | 27 | 7 | 17 | 14·5 | 18 | * | * | * | * | 4 | 48 |
| 3·9 | | | | | | | | | | | | | 52 | * | 15 | 40 | 4 | 34 |
| 3·8 | | | | | | | 11 | 28 | 12·5 | 45 | 8 | 21 | 8 | 50 | 7 | 45 | 5 | 23 |
| 3·7 | 4 | 1 | 2 | 1 | 6 | 2·8 | | | | | | | 6 | 30 | 5 | 3 | 5 | 10 |
| 3·6 | | | | | | | | | | | | 2 | 2 | 3 | 3 | 5 | 1 | 4 |
| 3·5 | | | | | | | 17 | 9·5 | 25 | 10 | 11 | 14·5 | | | | | | |
| 3·4 | | | | | | | | | | | | | | | | | | |
| 3·3 | | | | | | | | | | | | | | | | | | |
| 3·2 | | | | | | | 7·5 | 4·5 | 10 | 8·5 | 2 | 6·5 | | | | | | |

* Indicates no withdrawal of prostomium within the first minute after immersion. Figures are total seconds taken to withdraw. Threshold is taken as the first withdrawal time of 30 sec or less.

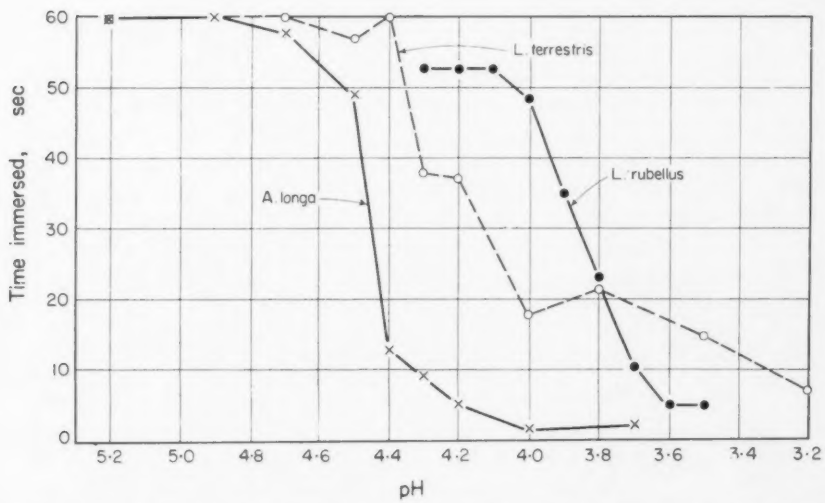


FIG. 1. Graph to show the average time taken to withdraw the prostomium from pH solutions, in three species of earthworms. × *A. longa*, ○ *L. terrestris*, ● *L. rubellus*.

At pH 4·3 and 4·2 the animals became more active, and withdrew after an average latent period of 38 sec. Stimulation with pH 4·0 reduced the latent period still further (18 sec) and still greater acidities showed similar latencies, though at pH 3·2 the length of the latent period had been decreased to 6·5 sec. These reaction times, and indeed the gross behaviour of *L. terrestris*, differed considerably from those of *A. longa*, withdrawal often occurring only after some time had been spent investigating the solution. Stimulation of the body wall may well have occurred at pH 4·3–4·2 such that the animal would not have burrowed into soil of this acidity, but the withdrawal response was not so violent as that of *A. longa* at this pH. Of the five *L. terrestris* two had thresholds (Table 1) at pH 4·3 and three at pH 4·2.

*L. rubellus*. When treated in the same way as *L. terrestris*, *L. rubellus* showed different reaction times. There was no sign of withdrawal of the prostomium at pH 4·3–4·1 but at pH 4·0, 3·9, 3·8 and 3·7, the length of time the prostomium remained in the solution diminished steadily. At pH 3·7, 3·6 and 3·5, the withdrawal was faster than that of *L. terrestris* (Table 1, Fig. 1).

The threshold of stimulation showed greater variation in the case of *L. rubellus* than in the other two species.

These simple dipping experiments tend to confirm the pot studies in that each species had a different tolerance towards pH. Thus *A. longa* was least tolerant of high acidity, whilst L. *terrestris* was more tolerant than *A. longa* but less so than *L. rubellus*. This is reflected by the speed of the withdrawal response under certain known pH conditions.

*Neurophysiology*

When buffer solutions are run against the body wall, as in the sodium chloride and quinine experiments described elsewhere (Laverack, 1960), a condition is set up that is similar to that existing when the animal is dipped into buffer solutions. The gross behavioural responses described above indicated that the threshold stimulation of *A. longa* occurred at pH 4·4, though the vigorous exploratory movements of the animals when dipped into buffer of pH 4·5 suggested that this pH might also be distinguished. *L. terrestris* and *L. rubellus* had apparent thresholds at pH 4·3–4·1 and pH 4·0–3·8 respectively.

In order to ascertain more precisely the threshold pH of the various earthworm species an examination of the sensory activity in the segmental nerves was carried out.

*A. longa*. Fig. 2 graphs a record made from a whole segmental nerve in segment 10 of *A. longa*. At pH 4·6 no alteration was noted in the slight spontaneous activity already present in the nerve. The application of pH 4·5 buffer, however, caused an immediate discharge in the nerve as sensory fibres were stimulated. A similar response was noted to pH 4·4 solutions. A number of nerve fibres were active and the maximum level of activity (in such a preparation) occurred at the beginning of the volley of action potentials, gradually decreasing as the sense organs adapted.

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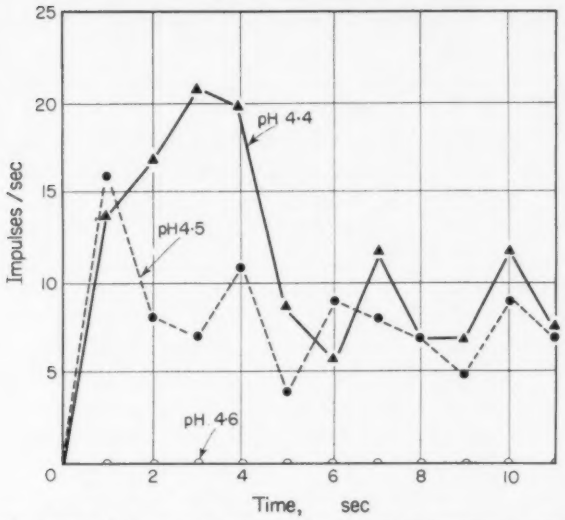


FIG. 2. Graph prepared from a photographic record of impulses monitored from a segmental nerve of *A. longa* in response to pH 4·5 (●) and pH 4·4 (▲). pH 4·6 (○) was without effect.

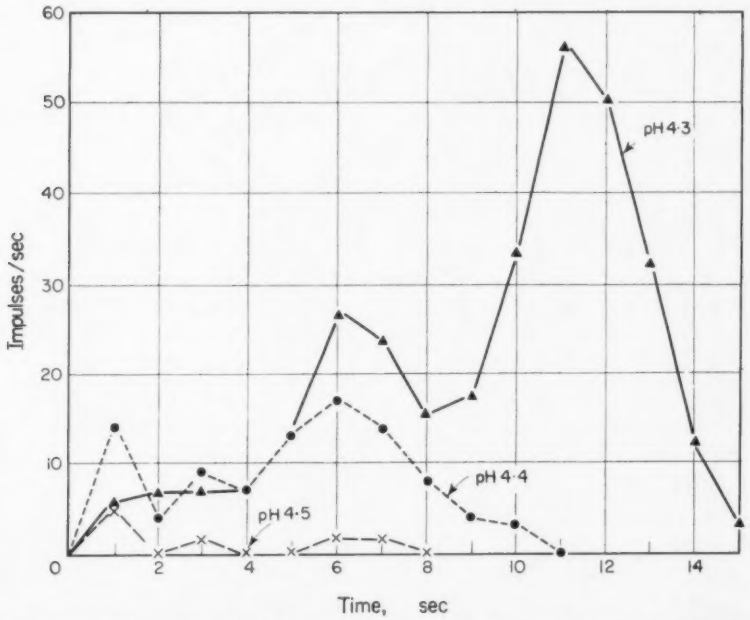


FIG. 3. Activity in a few-fibred preparation of *A. longa* segmental nerve to pH 4·5 (×), pH 4·4 (●) and pH 4·3 (▲). Note the gradually increasing frequency of impulses at pH 4·4 and pH 4·3. Threshold in this preparation was shown by the occurrence of a single potential at pH 4·6 (not plotted).

It has proved possible, by teasing, to isolate a few fibres that respond only to changes in the hydrogen ion concentration. Fig. 3 is a record of the activity in a pared segmental nerve in response to pH 4·5, 4·4 and 4·3. A buffer of pH 4·6 caused only a single potential to discharge and this would have been overlooked in a record from an intact nerve as the spontaneous background activity would have effectively camouflaged one spike. At pH 4·5 a burst of five action potentials occurred immediately after addition of the buffer, and these were followed by a few irregular spikes within the next few seconds. pH 4·4 and 4·3 both induced volleys of potentials that lasted for many seconds.

This level of stimulation in this preparation led to a progressive increase in frequency of the potentials. At pH 4·3 the initial frequency was 5/sec; after 4 sec it was 8/sec, and after 11 sec it was 57/sec. This burst then terminated abruptly and the nervous activity returned to the original level, though in a few cases a further, but shorter, burst was observed to occur some seconds after the first. Thus two forms of response have been observed. In the first of these the sensory nerves produced potentials swiftly at first and then gradually adapt to the stimulus, with a concurrent drop in frequency and eventual cessation of impulses. In the second case there was a gradually mounting frequency of impulses, terminating abruptly after reaching a high frequency.

Organs sensitive to the addition of acid stimuli in the pH range 4·6–4·4 and below are found to be universally distributed over the body surface of *A. longa*. There is no indication that the prostomium is more sensitive than the rest of the body wall. No other type of chemical stimulation had an effect on these fibres.

The threshold values for these fibres in *A. longa* varied slightly from individual to individual. In only one case did the initial response occur below pH 4·5, and in the majority of cases the nerves were already very active at this level indicating that pH 4·6 is more probably the common threshold as indicated above. In twelve experiments the threshold was pH 4·6 in six, pH 4·5 in five and pH 4·4 in one.

*L. terrestris*. The spontaneous activity in the segmental nerves of *L. terrestris* was very similar to that of *A. longa*. The reaction to pH changes gave the same general pattern, but with somewhat differing threshold values. In ten examples from which records were obtained the threshold response was pH 4·3 in three cases, pH 4·2 in three cases and between pH 4·2 and pH 4·1 in the remainder. In no case was the threshold below pH 4·1.

Figs. 4 and 5 show the reactions of two different preparations. In Fig. 4 taken from an intact segmental nerve there was no response to pH 4·4, but a distinct discharge of sensory fibres was seen upon the addition of pH 4·3, and increasing activity was noted in all subsequent records down to pH 3·8. Adaptation proceeds slowly in all cases.

Fig. 5, on the other hand, shows responses in a segmental nerve in which the addition of pH 4·2 to the body wall caused the discharge of only a single impulse after a latent period of 4 sec. pH 4·1 invoked a small burst, followed by regular discharge in one unit which lasted for some seconds. Rather greater and more prolonged bursts are noted in response to pH 3·8.

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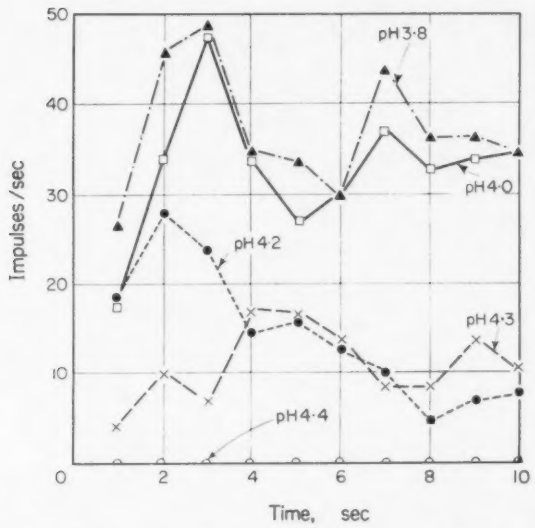


FIG. 4. Record from *L. terrestris* indicating threshold response at pH 4·5, followed by increasing activity with decreasing pH. No activity was noted in response to pH 4·4. ▲ pH 3·8, □ pH 4·0, ● pH 4·2, × pH 4·3, ○ pH 4·4.

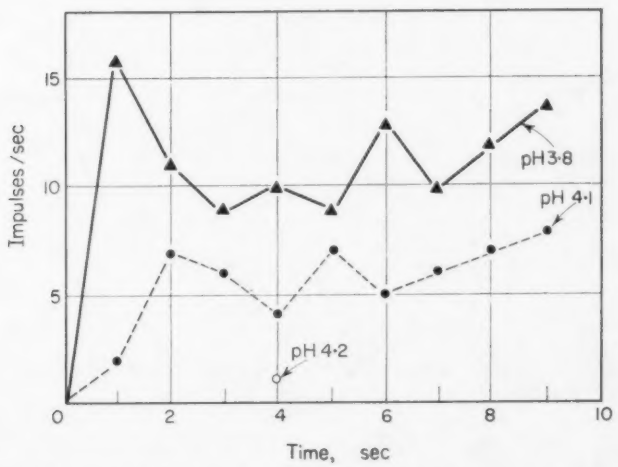


FIG. 5. A teased segmental nerve of *L. terrestris* with only a few sensory fibres responding to changing pH. A threshold of pH 4·2 is indicated by the single spike; pH 4·1 invoked a massive discharge. ○ pH 4·2, ● pH 4·1, △ pH 3·8.

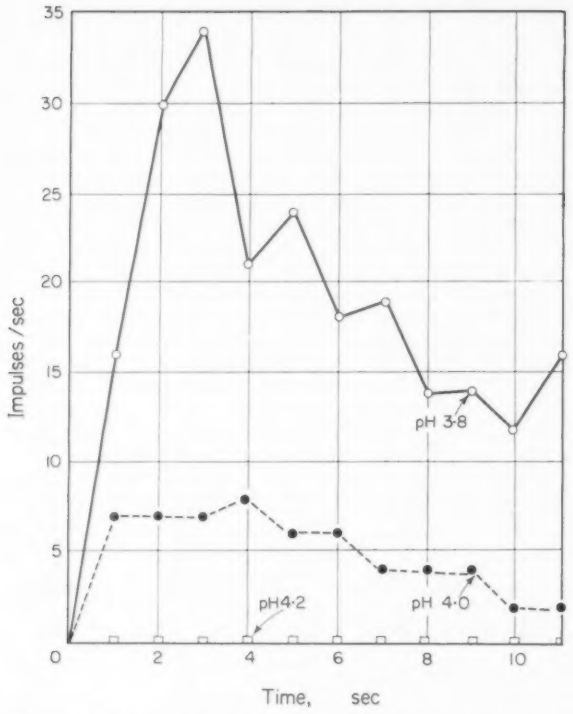


FIG. 6. A single unit of *L. terrestris* which gave potentials in response to pH 4·0 (●) and pH 3·8 (○), but not to pH 4·2 (□).

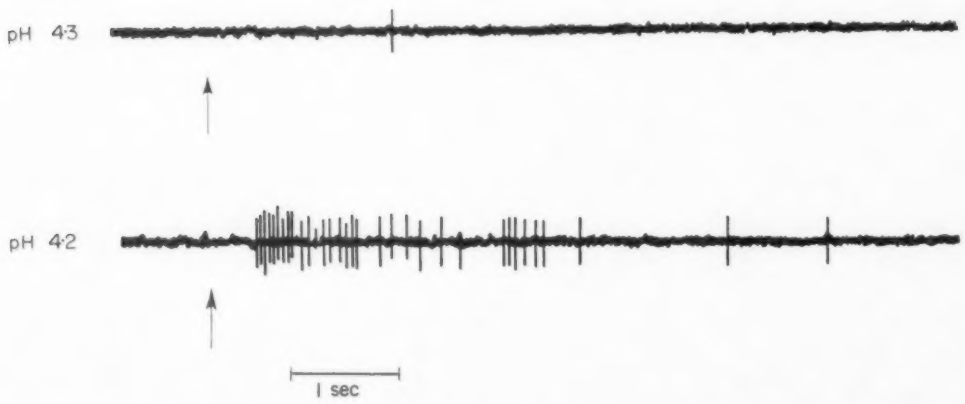


FIG. 7. Tracing of a record of a single fibre response in *L. terrestris* with a threshold at pH 4·3.

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Single active units have also been observed in this species by teasing to obtain thin strands of the segmental nerve. Fig. 6 shows that one such unit was quite strongly stimulated by pH 4·0, but no action potentials were seen in response to pH 4·2. It is thus assumed that the threshold of this fibre was ca. pH 4·1; pH 3·8 evoked a long discharge. Fig. 7 is a tracing of a photographic record showing the variation observed between individual animals, in that pH 4·3 caused a single sensory potential and pH 4·2 a short discharge.

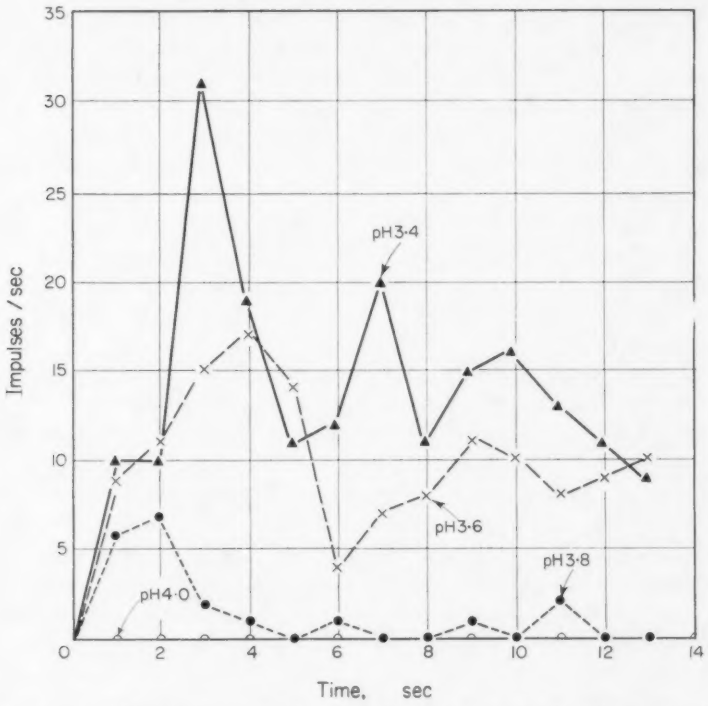


FIG. 8. Segmental nerve response (*L. rubellus*) to a range of acid values. ○ pH 4·0, ● pH 3·8, × pH 3·6, ▲ pH 3·4.

*L. rubellus*. This species is acid-tolerant and as such might be expected to have a threshold lying at a more acid pH than *A. longa* and *L. terrestris*. This has been found to be the case.

Fig. 8 was obtained from an unthinned segmental nerve of *L. rubellus*. A solution of pH 4·0 applied to the external surface was without effect upon the nervous activity, but pH 3·8 caused a slight increase in the activity and pH 3·6 and pH 3·4 both caused large increases in the activity of these nerves. It would seem, therefore, that the threshold for *L. rubellus* lies at approximately pH 3·8 and that further increases in acidity involve greater nervous response. Adaptation was slow when

the stimulus was intense. *L. rubellus* is a small species, and only four individuals were satisfactorily investigated. These gave threshold responses at pH 3·9 (one) and 3·8 (three).

## DISCUSSION

The field distribution and ecology of the various species of earthworms has been shown by statistical methods to be correlated with a number of variable factors in the soil. Among these variables it has been argued that pH is the most important (Satchell, 1956), or that calcium, due to its deficiency in acid soils, is the prime factor (Jefferson, 1956). In the latter case the calcium content of acid soils is low and it is suggested that because of this the growth of green plants and micro-organisms is limited, and thus the availability of food determines the size of earthworm populations. It has been known for many years (Robertson, 1936) that earthworms secrete calcium carbonate from the calciferous glands of the gut, but the precise role played by this system in earthworm metabolism is still uncertain. It appears, however, that the major part played by calcium in the bodily economy of earthworms will depend upon long-term deficiency effects leading to death of individuals, and eventually the population. This does not preclude continual recolonization of low-calcium sites from elsewhere so that a low-density population might always be present.



Satchell (1956), on the other hand, suggested that the effects of calcium deficiency, while doubtless of great importance, are mediated through the alliance with a low pH and that it is acidity which directly influences the distribution of earthworms. In particular, hydrogen ion concentration of pH 4·5 and below is of great importance. Bodenheimer (1934), basing his conclusions on ecological and experimental findings on the survival of *Allolobophora samarigera* concluded that pH was only limiting at extremes of alkalinity and acidity, particularly above pH 8 and below pH 5. Both Hurwitz (1910) and Arrhenius (1921) showed that acids would cause repulsion of earthworms, and this led to the conclusion that earthworms would flourish only in soils near neutrality, but gave no indication of the range of acidity that could be tolerated.

When specimens of *A. longa* are placed on soils not normally carrying a population of this species (e.g. soils at pH 4·1) an obvious behavioural response occurs. The animals will not burrow. They insinuate themselves into cracks and crevices if such are available, and these they proceed to line with mucus secretions, but if the soil is tamped hard they will not actively burrow into it. Instead they show considerable motor activity and carry out extensive exploration of the surroundings. Evidently some bodily stimulation occurs which is quickly acting, irritating, and does not require the ingestion of soil. If the animals are unable to escape from such a situation they cease exploration, cover the ground with mucus and remain quiescent.

Active withdrawal of earthworms at this pH (4·1) could not be shown by Satchell (1956), however, when he dipped the anterior segments of four species *A. nocturna*, *L. terrestris*, *E. rosea* and *A. caliginosa*, into sulphuric acid solutions

having different pH values. The first withdrawal of the prostomium of these earthworms was observed at pH 2·8, which, as Satchell pointed out, is a whole pH unit below the limiting values noted in field distribution. These experiments have now been repeated using buffer solutions instead of sulphuric acid. In contrast to the findings of Satchell it was noted the rejection of solutions occurred at pH 4·4 and below in the case of *A. longa*, pH 4·3–4·2 for *L. terrestris* and pH 3·9–3·8 for *L. rubellus*. Increased searching activity at pH values slightly above these figures has been taken to indicate that the animals are sensitive to these levels, although immediate repulsion does not take place.

An objection that might be raised regarding the use of buffer solutions in these experiments is that the animal responds to the separate constituent chemicals and not to pH—but if this was so then reaction would be expected to reach either a maximum or minimum at pH 4 (the highest concentration of potassium hydrogen phthalate) and increase or decrease on one or both sides of that pH. But as activity commences at specific pH values for each species and then increases steadily as the hydrogen ion increases it seems that the hydrogen ion is the only important variable in the buffers.

These behavioural experiments show that *A. longa* (found naturally in soils above pH 4·5) is in fact actively repelled by pH 4·4, and appears to be sensitive to pH 4·5; *L. terrestris*, slightly more tolerant in field conditions since it occurs down to pH 4·1, is also more tolerant in these experiments, while *L. rubellus* is relatively acid-tolerant and is not affected by pH 4·0.

These preliminary conclusions can be supported by neurophysiological experiments. Prosser (1934) showed that the body wall of *L. terrestris* was sensitive to acid solutions, particularly to hydrochloric acid, and that action potentials could be recorded in the segmental nerves in response to such stimulation. The buffer solutions employed in the present work enabled a range of pH differing in steps of only 0·1 unit to be used (where appropriate). The first impulses in acid sensitive fibres occurred at different pH values in different species. The threshold for stimulation in *A. longa* lay between pH 4·6 and 4·4, values above this range being non-stimulating (as was distilled water at pH 5·2), *L. terrestris* had a threshold at pH 4·3–4·1 and *L. rubellus* at pH 3·8.

These differing threshold values reflect closely the limits of distribution in the field of populations of these three species. *A. longa* has the highest pH threshold in experimental studies, the first stimulation occurring at pH 4·6–4·4. In nature, earthworms of this species are not found below pH 4·5. *L. terrestris* showed threshold values of pH 4·3–4·1 and is not normally obtained from soils below pH 4·2, while *L. rubellus* is a ubiquitous form capable of living in sites of pH 3·7 (Satchell, 1956), and showing in this study a pH threshold at pH 3·8.

It is apparent, therefore, that there are distinct differences between species of earthworms in their ability to detect and react to hydrogen ion concentration. The field distribution of the different species reflects the varying sensitivities of their sense organs. Weakly stimulating acidities would lead to exploratory and rejection behaviour, whilst intense stimulation by very low pH would cause violent reaction,

presumably by bringing the giant fibre system into action. The lack of calcium in such sites would seem to be of secondary importance since the immediate avoidance of such soils would mean that there is no opportunity for lack of calcium to play a decisive role through nutrient deficiency.

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# POLYPHENOLS AND THEIR QUINONE DERIVATIVES IN THE CUTICLE OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA* (FORSKÅL)

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**Abstract**—(1) Several *ortho*-dihydroxyphenols, both deaminated and undeaminated, are present in the abdominal cuticle of adult *Schistocerca* both before and after it has naturally hardened. They are thought to be derived from the phenolic substance formed in the blood by the enzymatic oxidation of tyrosine.

(2) It is shown that degradation of *ortho*-dihydroxyphenols takes place in the cuticle, and is accompanied by decarboxylation.

(3) No *para*-dihydroxyphenols are present, however, their absence being due to the fact that the cuticle is incapable of hydroxylating the naturally occurring aromatic amino acids.

(4) It is shown, in conclusion, that the colourless and unresistant exocuticle of *Schistocerca*, which differs markedly from that of darkly coloured insects i.e. *Periplaneta* (Kennaugh, 1958), is tanned only by *ortho*-quinones.

## INTRODUCTION

KENNAUGH (1958) has recently found that the hardening and darkening of the exocuticle of the cockroach *Periplaneta americana* is due to the same processes as take place in the formation of the puparium of the blowfly *Calliphora vomitoria* (Dennell, 1958c). The tanning of the cuticular substrate was regarded as chiefly due to *para*-benzoquinone produced by the oxidation of hydroquinone, and not to *ortho*-quinones as had previously been thought (Dennell & Malek, 1955; Dennell, 1947). The dihydroxyphenol formed in the blood which had formerly been regarded as the only source of tanning quinones was considered to be responsible primarily for melanin formation in the cuticle.

Hitherto, only *ortho*-dihydroxyphenolic acids have been found in the hard cuticle of the desert locust, *Schistocerca gregaria* (Malek, 1957). The question now arises whether *para*-dihydroxyphenols may be involved in cuticle hardening as in *Periplaneta* and *Calliphora*.

The observations on which this paper is based have been confined to the abdominal cuticle of adult locusts. As already described (Malek, 1958a), the cuticle of this region is uniformly colourless, and no confusion therefore arises due to the presence of melanin or its precursors.

The methods and techniques adopted in this work are conveniently mentioned in the text.

## I. CHANGES IN POLYPHENOL CONTENT OF THE DEVELOPING CUTICLE

Although it has been repeatedly shown that dihydroxyphenols occur in both soft and hard cuticles of insects (Wigglesworth, 1957), it was not until recently that a change in the polyphenol content of the developing cuticle was demonstrated. Dennell (1958c), to whom this observation is due, found that the *para*-dihydroxyphenol hydroquinone is present, among other hydroxy acids, in the larval cuticles of *Calliphora* but not in the cast puparia. On the basis of this and other evidence, and the fact that hydroquinone is readily oxidized by the cuticle, this substance was regarded as giving rise to the tanning quinone.

To determine what changes in polyphenol content take place in the developing cuticle of *Schistocerca*, a chromatographic examination of soft and fully hardened cuticles has been made. In preparing hard cuticles for analysis tergites and sternites from fifty sexually mature locusts were scraped free of adhering tissues, and exhaustively extracted with hot water under reflux. After filtering free from insoluble material, the aqueous extract was acidified with sulphuric acid and then shaken with three successive volumes of hydrogen peroxide-free ether. Before use the ether was freed by repeated distillation from hydroquinone which might be present as a stabilizer. On evaporating the ether extract under reduced pressure a yellow solid residue remained, and was taken up in 1 ml of 10 per cent *iso*-propyl alcohol. Preliminary tests showed the presence of phenolic reducing substances. The solution was then examined chromatographically using benzene/acetic acid/water (40 : 40 : 20), butanol/acetic acid/water (40 : 10 : 50) and *meta*-cresol/acetic acid/water (50 : 2 : 48) as solvents. Benzene/acetic acid/water gave by far the best separation, and revealed as many as nine substances reducing ammoniacal silver nitrate. It has not proved possible to identify all these substances. None, however, gave reactions characteristic of *para*-dihydroxyphenols. When tested with 1% ferric chloride solution all immediately developed an intense green colour, a characteristic of *ortho*-dihydroxyphenols, and gave a pronounced blue with the Folin-Ciocalteau reagent only after being made alkaline. Dalgliesh (1955) regards the second of these reactions as indicating the presence of *ortho*-diphenols, *para*-diphenols reacting without the addition of alkali.

Insomuch as no free *para*-dihydroxyphenols are present in the hard cuticles of *Schistocerca* these results are therefore in agreement with those of Dennell (1958c). This author, however, found that the cast puparia of *Calliphora* contain considerable amounts of pyrogallol. Being a trihydroxyphenol, this substance does not give a green colour with ferric chloride, and for this reason is not regarded as present in the hard cuticles of *Schistocerca*. Its presence in *Calliphora* but not in *Schistocerca* constitutes an important difference between the cuticles of these two insects which will be referred to later.

Only one of the silver-reducing substances revealed in the extract of the cuticles of *Schistocerca* gave a definite colour with ninhydrin, indicating the presence of free α-amino groups. It proved, on comparison with an authentic sample, to be 3 : 4-dihydroxyphenylalanine (I, Fig. 1). The occurrence of the same substance in fully hardened cuticles other than those of *Schistocerca* has already been

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demonstrated (Schmalfuss & Müller, 1927; Pryor *et al.*, 1947). Among the silver-reducing substances in the extract, which did not react with ninhydrin, the following phenols were found to be present by comparison with reference compounds: 3 : 4-dihydroxyphenylpropionic acid (II), 3 : 4-dihydroxyphenylacetic acid (III), 3 : 4-dihydroxybenzoic acid (protocatechuic acid) (IV) and catechol (V). The identity of these substances was further established by the fact that they gave the same reactions as the reference compounds when tested with the

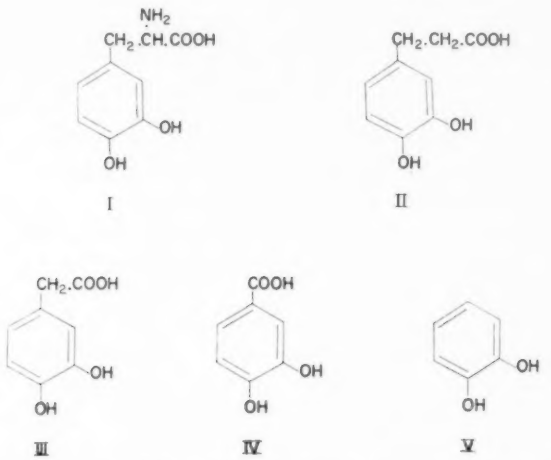


FIG. 1. Structural formulae of the polyhydric phenols present in aqueous extracts of the cuticle of *Schistocerca*. I–IV, in the soft cuticle; I–V, in the hard.
I, 3 : 4-dihydroxyphenylalanine; II, 3 : 4-dihydroxyphenylpropionic acid; III, 3 : 4-dihydroxyphenylacetic acid; IV, 3 : 4-dihydroxybenzoic acid; V, catechol.

phenol reagents found useful by Dennell (1958a). These reagents are: a solution of phloroglucinol in 10% sodium hydroxide, Ehrlich's "strong" reagent and the sucrose reagent of Roux. It will be observed from the formulae given in Fig. 1 that the phenolic compounds present in the cuticle are so closely related to each other as to suggest their derivation from a common source. Richards (1951) has already suggested that they may represent intermediate stages in the deamination and degradation of the blood phenol 3:4-dihydroxyphenylalanine. But whether they are truly derived in this way or are directly produced in the cuticle by non-specific hydroxylation (Dennell, 1958b) awaits further investigation. The presence among them of catechol is important, because this compound has for many years been regarded as the tanning phenol of insect cuticles generally (Mason, 1955), its production being envisaged from the related phenol protocatechuic acid which has been identified in a wide variety of insects (Pryor *et al.*, 1947; Hackman *et al.*, 1948). It may therefore be significant that both catechol and protocatechuic acid occur together in the cuticle of *Schistocerca*. Catechol has previously been recognized in insect cuticles by Dennell (1958c) and Kennaugh (1958). The

presence of 3 : 4-dihydroxyphenylacetic acid in other insect cuticles has already been demonstrated by Pryor *et al.* (1947) and Hackman *et al.* (1948). 3 : 4-dihydroxyphenylpropionic acid does not seem, however, to have been hitherto reported.

Attention was next directed to the polyphenols present in soft cuticles of *Schistocerca* when newly exposed on moulting. The problem of collecting adequate material for the extraction was to some extent simplified by keeping a large stock of last instar nymphs; whenever these moulted to the adult stage their cuticles were scraped clean, and at once placed in the hot extractor in order to inhibit the cuticular oxidase. In total, fifty abdominal cuticles were obtained and treated in the manner described above for the hard cuticles. It was expected that they would yield a high concentration of free polyphenols already present in preparation for the hardening process. This was not found to be so, the extract being only faintly reducing. This indicates that the tanning phenols are mainly present in the cuticle only at a later stage during hardening. To facilitate the chromatographic examination of the extract from soft cuticles its volume was further reduced, but it was not until unusually large amounts of the concentrated extract were applied to the chromatograms that the presence of the following phenols became established: 3 : 4-dihydroxyphenylalanine (I), 3 : 4-dihydroxyphenylpropionic acid (II), 3 : 4-dihydroxyphenylacetic acid (III) and 3 : 4-dihydroxybenzoic acid (IV). Except for the differences in concentration noted above, and the absence of catechol, these phenols are those which were found in the hard cuticles. The absence of catechol is remarkable, and suggests that it is produced in the cuticle sometime after moulting has taken place. This is in marked contrast to the events in *Calliphora* (Dennell, 1958c) where catechol is present in the soft larval cuticle but not in the hard puparium.

The facts that hydroquinone was not recognized in aqueous extracts of soft or hard cuticles, and that all the phenols identified are related 3 : 4-dihydroxy compounds derivable from a common source, suggest that non-specific hydroxylation of aromatic amino acids (Dennell, 1958b; Kennaugh, 1958) does not take place in *Schistocerca*. This will be further discussed later.

Phenolic substances other than those mentioned above were also found in the extract of the soft cuticles but were not identified. As already stated they were all *ortho*-dihydroxyphenols. One of them, however, showed peculiar properties and is worth reporting on. It had an $R_f$ value, in benzene/acetic acid/water, of 0·04, and of 0·73 in *meta*-cresol/acetic acid/water. Although it developed a blue colour with the Folin-Ciocalteau reagent before being made alkaline, suggesting that it might be a *para*-dihydroxyphenol, it gave very strongly the green colour characteristic of *ortho*-dihydroxyphenols when tested with ferric chloride. This substance is evidently not hydroquinone, which fails to react with ferric chloride. Its reactions given with other phenol reagents, which are an intense brown colour with alkaline phloroglucinol and a pink one with the sucrose reagent of Roux, add further to its distinction from hydroquinone. To the objection that a mixture of two or more phenols rather than a single substance is present, it may be answered that this

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compound remained unseparated in the different solvents used, and gave identical reactions after each treatment. It appears, however, that the reactions given with the Folin-Ciocalteau reagent are not specific to any particular type of phenol, for it has been found in this work that *meta*-cresol responds towards this reagent as do *ortho*-dihydroxyphenols, and further that catechol and its derivatives, in spite of being *ortho*-dihydroxyphenols, develop a blue-green colour in the absence of alkali which is hardly distinguished from a blue one given under the same conditions with *para*-dihydroxyphenols. Whatever its nature may be, this compound is present in the soft and in the hard cuticles alike, and apparently in equal concentrations.

## II. EXAMINATION OF THE QUINONE–PROTEIN CONJUGATE OF THE HARD CUTICLE

### (a) *The effect of alkaline hydrolysis*

The evidence obtained so far merely shows that *ortho*-dihydroxyphenols are the only phenols which occur freely in the cuticle of *Schistocerca*, before as well as after it has been hardened. The existence of these compounds in the cuticle does not, of course, establish their function in sclerotization. Nor does the absence of free hydroquinone from the cuticle necessarily eliminate the possibility of its participation in this process.

To test this matter further it was decided to try to identify the quinone combined with protein in the hard cuticle. Before this could be successfully accomplished it was necessary to separate the quinone from the protein by disrupting the intervening bond. Dennell (1958c) found that the akaline stannite reagent used by Lugg (1938) (a mixture of sodium hydroxide and stannous chloride) is capable of bringing about this effect, and further of subsequently reducing the detached quinone to its parent polyphenol. Cast puparia of *Calliphora* (Dennell, 1958c) and hard cuticles of *Periplaneta* (Kennaugh, 1958) treated in this way yielded a ninhydrin-positive *para*-dihydroxyphenol. This compound has been identified as an amino hydroquinone.

The fully hardened cuticles of *Schistocerca* remaining after all free phenols were removed by extraction with water in the previous experiment were treated with the alkaline stannite reagent under the conditions prescribed by Lugg (1938). After 24 hr the golden yellow hydrolysate was acidified with sulphuric acid, when its colour was completely discharged. This is important, for the pigments formed from *ortho*-benzoquinone and amino acids have this property, but not those formed from *para*-benzoquinone (Mason, 1955). The alkaline stannite hydrolysate of *Calliphora* puparia does not change colour on acidification (Dennell, personal communication). Extraction of the acidified hydrolysate with ether and subsequent evaporation of the solution yielded a pale straw-coloured residue. In contrast with that obtained under similar conditions from *Periplaneta* (Kennaugh, 1958) and *Calliphora* (Dennell, 1958c), this residue did not change colour on exposure to air, indicating further that it may be of different composition. This indication was supported when a solution of the residue in 10% *iso*-propyl alcohol was

examined chromatographically. Several silver-reducing spots were revealed, but none of them gave reactions similar to those given by hydroquinone. Instead, they all developed an intense green colour with ferric chloride, and became distinct blue with the Folin-Ciocalteau reagent only after being made alkaline. It is therefore apparent that the substances responsible for these spots can only be *ortho*-dihydroxyphenols.

As in *Calliphora* and in *Periplaneta*, all the regenerated phenols were ninhydrin-positive. The lilac colour given with Ehrlich's reagent supports the view that they are aminophenols. Dennell (1958c) has suggested that the amino groups attached to hydroquinone which he recovered from *Calliphora* puparia were derived from the protein which has been tanned, being those involved in combination with the quinone. If Dennell's view is well founded, as it appears to be, then the substances obtained from hard cuticles by hydrolysis and reduction may be regarded as being derived from *ortho*-dihydroxyphenols by way of *ortho*-quinones. On this basis *ortho*- and not *para*-dihydroxphenols are to be regarded as responsible for tanning the cuticle of *Schistocerca*. It therefore seems clear that although the cuticle of *Schistocerca* agrees with those of *Periplaneta* and of *Calliphora* puparia in being phenolically tanned, it differs as to the nature of the tanning agents involved.

Attention was next directed towards the identification of the aminophenols obtained from the cuticle of *Schistocerca* after the alkaline stannite treatment. In the absence of reference compounds their identity could not be established beyond doubt at this stage, but examination in ultra-violet light, together with the application of the reagents for the detection of polyphenols already referred to, indicated the presence of compounds corresponding in $R_f$ values and colour reactions with the following *ortho*-dihydroxyphenols: 3 : 4-dihydroxyphenylpropionic acid, 3 : 4-dihydroxyphenylacetic acid, 3 : 4-dihydroxybenzoic acid and catechol. Those corresponding to the first three compounds were present in abundant amounts, and that to catechol in only small amounts. It appears that the regenerated phenols, although carrying one or more amino groups, have the same $R_f$ values as their corresponding deaminated forms when used as standards. Direct evidence for the identity of these compounds is, however, presented later, when it will be shown that identical ninhydrin-positive substances are obtained from cuticles which have been artificially tanned with the above *ortho*-dihydroxyphenols.

In addition to the above amino compounds many others were also regenerated from the fully hardened cuticles. Only two of them were, however, present in high concentration, but their nature was not realized until later during this work. It will be merely stated at this stage that they proved to be derivatives of two of the above identified compounds. The remaining compounds which were present in low concentration were not identified.

(b) *The effect of acid hydrolysis*

If all the aminophenols obtained by alkaline hydrolysis and reduction in the previous experiment were truly derived from tanned protein, and were not merely trapped in its substrate, then none of them ought to be yielded if hydrolysis of hard

cuticles is performed under acid conditions without reduction. Acid hydrolysis does not disrupt the bond between quinone and protein (Hackman & Todd, 1953) as does alkaline hydrolysis. Hydrolysis with acid was therefore carried out with the aim of throwing further light on the origin of the above phenolic compounds which appear during alkaline stannite treatment.

Hard cuticles of *Schistocerca* were exhaustively extracted with hot water to remove free dihydroxyphenols, and then hydrolysed in 6 N HCl under reflux. The cuticles dissolved almost completely in the acid in less than 1 hr, but hydrolysis was continued for 24 hr. After filtration the dark brown hydrolysate was extracted with ether in the usual way. Chromatograms of the extract were developed in benzene/acetic acid/water (40 : 40 : 20) and sprayed with ninhydrin or ammonical silver nitrate. The results were unequivocal. Although three phenols were revealed, none of them gave reactions which indicated the presence of attached amino groups. It must therefore be concluded that treatment of hard cuticles with the alkaline stannite reagent, as has been done in the previous experiment, does indeed free the tanning phenols. Dennell (1958c) found that the amino hydroquinone which he obtained from *Calliphora* puparia by alkaline hydrolysis and reduction was not yielded when puparia were treated with sodium hydroxide alone. He therefore concluded that the action of the sodium hydroxide consisted merely of disrupting the bond between quinone and protein, while the addition of stannous chloride reduced the detached quinone. The present observation that no amino-phenols are yielded after acid hydrolysis adds further support to Dennell's conclusion.

Further examination of the three phenols obtained after acid hydrolysis in the present experiment indicated that they were *ortho*-dihydroxyphenols. Two of them proved, on comparison with authentic samples, to be protocatechuic acid and 4-methylcatechol. The nature of the third phenol remains unknown.

How these deaminated phenols are bound in the hard cuticle, so that they are not yielded on extraction with water, is not clear. They may be adsorbed to protein or bound to quinone in a manner similar to that proposed by Mason (1955) for the binding of quinone to collagen independently of amino or hydroxyl groups. Hitherto, phenols have only been shown to occur in an insect cuticle either free in a readily extractable form or combined with protein as quinones.

## III. THE ABSENCE OF A NON-SPECIFIC HYDROXYLATING SYSTEM FROM THE CUTICLE

It has already been shown that the *para*-dihydroxyphenol hydroquinone is absent from the soft cuticles of *Schistocerca*. Nor does its corresponding quinone occur in them after they have been hardened. In view of the importance that has been attached to this phenol in hardening the cuticles of *Periplaneta* and of *Calliphora* puparia as discussed above, it was decided to try to discover the reason for its non-production in *Schistocerca*. Dennell (1958b) has shown that this phenol, unlike the *ortho*-dihydroxyphenol formed in the blood, is produced in the larval cuticle of *Calliphora* in a very direct manner by the non-enzymatic hydroxylation

of the naturally occurring aromatic amino acids. Under experimental conditions the cuticle was found to be capable of hydroxylating such substrates as phenylalanine, *o*- and *p*-tyrosine and tryptophan. Dennell's conclusions in *Calliphora* were supported by those of Kennaugh (1958), who found in *Periplaneta* the same evidence for the presence in the soft cuticles of a similar non-specific hydroxylating system. Whether it is the absence of this system or of an appropriate substrate from the cuticle of *Schistocerca* which is responsible for the absence of hydroquinone remains to be investigated.

Soft abdominal cuticles obtained from six locusts which moulted at the same time were scraped free of adhering tissues. They were washed in several changes of distilled water at 37°C to remove free phenols and as much as possible of the substrates which might be naturally present. The cuticles were then incubated and aerated at 27°C in 10 ml of a 0·05% solution of phenylalanine. This concentration was recommended by Kennaugh (1958), who has found that the use of higher concentrations tended to obscure the chromatograms. After incubation for 48 hr neither the cuticles nor the solution underwent any noticeable change in colour. This observation is noteworthy, for both Dennell (1958b) and Kennaugh (1958) reported considerable darkening when larval cuticles of *Calliphora* or soft cuticles of *Periplaneta* were incubated under similar conditions. After the cuticles were removed, the incubation medium was evaporated to dryness in a vacuum desiccator, and the residue redissolved in 1 ml of 10% *iso*-propyl alcohol. This solution was examined chromatographically using butanol/acetic acid/water (40 : 10 : 50) and benzene/acetic acid/water (40 : 40 : 20) as solvents. Location of spots was effected by examination under ultra-violet light followed by spraying with ninhydrin or ammoniacal silver nitrate. In addition to the phenylalanine used as the substrate eight other spots positive to ninhydrin, one of which very faintly reduced ammoniacal silver nitrate, were found. One of the spots positive to ninhydrin was identified as *p*-tyrosine, and that corresponding with the silver-reducing spot proved to be 3 : 4-dihydroxyphenylalanine. However, similar results were obtained when water alone was used in place of phenylalanine to incubate the cuticles, showing that 3 : 4-dihydroxyphenylalanine was not derived from phenylalanine. In view of the fact that a phenol oxidase occurs in the cuticle at this stage (Malek, 1957), it was suspected that the production of 3 : 4-dihydroxyphenylalanine under these conditions might be due to the enzymatic hydroxylation of *p*-tyrosine present in the cuticle and recovered from the incubation media. That this is true was shown by the fact that this diphenol was not produced in the presence of oxidase inhibitors such as cyanide or phenylthiourea.

It therefore seems clear that the cuticle of *Schistocerca* is incapable of hydroxylating phenylalanine under experimental conditions. *p*-Tyrosine, on the other hand, is hydroxylated to 3 : 4-dihydroxyphenylalanine, but only in the presence of the cuticular oxidase. These observations are in marked contrast with those of Kennaugh (1958) and of Dennell (1958b), who found that incubation of soft cuticles of *Periplaneta* and of larvae of *Calliphora* in *p*-tyrosine resulted in the

production of hydroquinone. Failure to detect hydroquinone in the present experiments must be regarded as indicating that a non-specific hydroxylating system is absent from the cuticle of *Schistocerca*.

This conclusion is supported by the fact that the cuticle of *Schistocerca* does not contain pyrogallol (see p. 36). Like hydroquinone, this phenol is present in the larval cuticles of *Calliphora*, and Dennell (1958b) has given evidence that the same mechanism is responsible for its production as for hydroquinone. Pyrogallol is a trihydroxyphenol and in the absence of a similar hydroxylating mechanism is not expected to be found in *Schistocerca*.

Not only is hydroquinone not produced in the cuticle of *Schistocerca*, but it is not oxidized by the cuticle. When white cuticles of newly moulted locusts were immersed in a 1% solution of hydroquinone they remained uncoloured even after 48 hr. This result was not unexpected, of course, as this phenol is not regarded as a substrate for polyphenol oxidases. But the ease with which the same substance is oxidized by the larval cuticle of *Calliphora* is remarkable (Dennell, 1958c). This observation has been repeated in this work and confirmed. It has been explained on the basis that the *ortho*-diphenol catechol, which is present in the larval cuticle in addition to hydroquinone, is first oxidized by the cuticular oxidase, and that the resulting quinone subsequently oxidizes hydroquinone to *para*-benzoquinone. But catechol is not found in the soft cuticles of *Schistocerca*, so that hydroquinone, even if it were naturally present in the cuticle, would presumably remain unoxidized.

The important outcome of the above observations is, first, that different cuticles of insects may contain different phenolic compounds and, secondly, that the ability of cuticles to utilize phenolic substrates also differs.

Turning now to the *ortho*-dihydroxyphenols which are naturally present in the cuticle of *Schistocerca*, it seems that the only route for their production is the deamination and degradation of the dihydroxyphenol formed in the blood by the specific action of tyrosinase on tyrosine. The blood of newly moulted locusts, like that of other insects (Dennell, 1947; Dennell & Malek, 1955), readily darkens on exposure to air, indicating the presence of active phenolase. Although the true nature of the insect blood phenol has never been demonstrated, it is universally thought to be 3 : 4-dihydroxyphenylalanine (Pryor, 1940; Dennell, 1947; Fraenkel & Rudall, 1947), which has been found in both the soft and hard cuticles of *Schistocerca*. Fraenkel & Rudall (1947) have suggested that the deamination of this compound might not be complete, so that under certain conditions there may be a degree of protein tanning by products of deaminated tyrosine as well as formation of melanin by undeaminated products. No melanin is formed in the abdominal cuticle of adult *Schistocerca*, and the 3 : 4-dihydroxyphenylalanine remaining in them after they have been hardened may be regarded as representing that portion originally intended for melanin formation. This suggestion may be supported by the fact that cast exuviae of nymphs of *Schistocerca*, which are highly melanized, have not been found in this work to contain free 3 : 4-dihydroxyphenylalanine. There is little room for doubt that the 3 : 4-dihydroxyphenylalanine of the

blood is the precursor of melanin in the cuticle. But in addition in *Schistocerca* it seems the only source of the tanning phenols, unlike the condition in blowfly larvae.

## IV. THE IDENTITY AND METABOLISM OF THE TANNING PHENOLS

It now seems evident that the cuticle of *Schistocerca* is tanned by *ortho*-quinones, and the recovery of the amino derivatives of 3 : 4-dihydroxyphenylpropionic acid, 3 : 4-dihydroxyphenylacetic acid, protocatechuic acid and catechol after alkaline stannite treatment suggests that the corresponding non-aminated diphenols are those which give rise to the effective quinones. All are found in the fully hardened cuticle.

But it still seems desirable to show that cuticles tanned with these diphenols in an artificial manner yield the same amino compounds as those obtained from the naturally tanned cuticles after treatment with the alkaline stannite reagent. For this purpose, soft cuticles from about 200 newly moulted adult locusts were scraped clean and washed in several changes of distilled water to remove free phenols. They were divided into four equal batches, and each incubated and aerated at 27°C in a 0·1% solution of one of the following phenols: 3 : 4-dihydroxyphenylpropionic acid, 3 : 4-dihydroxyphenylacetic acid, protocatechuic acid and catechol. After incubation for 48 hr both the cuticles and the solutions had become strongly coloured, either red or dark brown, indicating that oxidation of the substrates used (or substances derived from them) must have taken place. The oxidation of protocatechuic acid under these conditions is remarkable, for Kennaugh (1958) and Dennell (1958c) have found that this phenol is not oxidized to any appreciable extent by the soft cuticles of *Periplaneta* or of *Calliphora* larvae. After the cuticles have been removed from the incubation media, they were first exhaustively extracted with hot water to remove the added substrates and derived substances, and then treated with the alkaline stannite reagent in the manner described earlier. The alkaline solutions remaining after this treatment resembled the hydrolysate obtained under similar conditions from the naturally tanned cuticles in being golden yellow, and further in losing this colour upon acidification. Still closer resemblance between these preparations was indicated when none of the phenolic residues which they yielded after extraction with ether changed colour on exposure to air. Each residue was taken up in 1 ml of 10% *iso*-propyl alcohol and examined chromatographically. The results were unequivocal. From each of the four extracts a silver-reducing substance was obtained which was ninhydrin-positive and which showed the same characteristics as one of the four aminophenols already identified in the naturally hardened cuticles. These results leave little doubt that the four *ortho*-dihydroxyphenols used as substrates in this experiment are truly involved in hardening the cuticle of *Schistocerca*. Although they appear to support the view already held by many earlier workers (Hackman *et al.*, 1948; Hackman & Todd, 1953) that an insect cuticle may be tanned by *ortho*-quinones, no claim can be made that these compounds are commonly occurring tanning agents until the cuticles of many more insects have been

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examined. Furthermore, it is to be emphasized that the tanning of the cuticle of *Schistocerca* in this way results in the production of an exocuticle very different from that of insects generally (p. 47, Table 2).

Further analysis of the results shown by this experiment yielded valuable information on the way in which *ortho*-dihydroxyphenols are metabolized in the cuticle of *Schistocerca*. It has been found that those cuticles which had been artificially tanned with protocatechuic acid yielded amino derivatives of both this phenol and of catechol after treatment with the alkaline stannite reagent. The latter compound was recovered alone from cuticles tanned with catechol. These observations indicate that the oxidation and condensation of protocatechuic acid is accompanied by partial decarboxylation. Since both compounds were also yielded by the naturally tanned cuticles as described above, it became apparent that the same reaction takes place *in vivo* as does under experimental conditions. This explains why free catechol, which is not found in the cuticles when newly exposed on moulting, is present after they have been hardened. A closely similar reaction has been demonstrated by Hackman & Todd (1953) to take place when protocatechuic acid is incubated with mushroom tyrosinase. Under these conditions, however, protocatechuic acid was completely decarboxylated, so that in the presence of extracted cuticular protein it formed the same compound as when catechol was used. Dennell (1958c) reported the occurrence of catechol in the larval cuticle of *Calliphora*, but regarded it as derived not from protocatechuic acid in the manner described above but from phenylalanine as a result of non-specific hydroxylation.

Of far-reaching importance were the results of using 3 : 4-dihydroxyphenylacetic acid to tan soft cuticles. In addition to the amino derivative of this substance, those of protocatechuic acid and of catechol were also obtained after alkaline stannite treatment. These observations are intelligible if it is considered that the dihydroxyphenylacetic acid used as the substrate is first degraded to protocatechuic acid, and that the latter phenol is subsequently decarboxylated to catechol as suggested above. And just as protocatechuic acid is decarboxylated in the cuticle, the decarboxylation of 3 : 4-dihydroxyphenylacetic acid may similarly be effected independently of degradation. In that case it should yield 4-methylcatechol (homocatechol) which, like the other phenolic products, may condense with the cuticular protein. Examination of the chromatograms revealed the presence of a strongly reducing substance which had an $R_f$ value of 0·44 in benzene/acetic acid/water, and of 0·80 in *meta*-cresol/acetic acid/water. This reducing substance fluoresced blue in ultra-violet light, gave a lilac colour with ninhydrin, pink with the reagents of Ehrlich and of Roux, orange red with alkaline phloroglucinol and dirty green with ferric chloride. With the Folin-Ciocalteau reagent it developed a blue-green colour in the absence of sodium carbonate, and more intense blue after this alkali has been added. These properties, except for the ninhydrin reaction, are typical of an authentic sample of 4-methylcatechol. The same substance was obtained from the naturally tanned cuticles after alkaline hydrolysis under reducing conditions. It will be remembered that

4-methylcatechol itself was found after acid hydrolysis. No doubt is left that the cuticle of *Schistocerca* is capable of decarboxylating *ortho*-dihydroxyphenols.

The results obtained with 3 : 4-dihydroxyphenylacetic acid were strongly supported when the propionic derivative was used to tan soft cuticles. The degradation of this phenol during incubation may be expected to give rise first to 3 : 4-dihydroxyphenylacetic acid and then to protocatechuic acid. These products in turn should yield 4-methylcatechol and catechol when subsequently decarboxylated as has been shown above. In accordance with expectation amino

TABLE 1—THE PHENOLS PRODUCED BY THE CUTICLE OF *Schistocerca* DURING INCUBATION WITH VARIOUS *ortho*-DIHYDROXYPHENOLS

| Substrate | Phenols produced | | |
|---|---|---|---|
| | Catechol | Protocatechuic acid | 3 : 4-dihydroxyphenyl-acetic acid |
| Catechol | − | − | − |
| Protocatechuic acid | + | − | − |
| 3 : 4-dihydroxyphenylacetic acid | + | + | − |
| 3 : 4-dihydroxyphenylpropionic acid | + | + | + |

+ = present; − = absent.

derivatives of all these compounds were recovered after treatment with the alkaline stannite reagent. But, in addition, a ninhydrin-positive reducing substance with properties closely similar to those of 4-methylcatechol was also obtained. This reducing substance had, however, an $R_f$ value, in benzene/acetic acid/water, of 0·56, higher than that of 4-methylcatechol. It can only be presumed to be the amino derivative of 4-ethylcatechol similarly formed by the decarboxylation of the 3 : 4-dihydroxyphenylpropionic acid used as the tanning substrate.

Degradation of *ortho*-dihydroxyphenols to compounds having shorter side chains, accompanied by decarboxylation, is therefore shown to take place in the cuticle of *Schistocerca*. Hitherto, the occurrence of these processes in an insect cuticle has only been postulated (Richards, 1951).

Since it is now evident that *ortho*-dihydroxyphenols undergo degradation and decarboxylation in the cuticle of *Schistocerca* it may be expected that at least some of the products yielded by these processes remain in the substrate solutions after the cuticles have been artificially tanned. To test this possibility, the solutions were evaporated to dryness in vacuum desiccators over phosphorous pentoxide. The coloured residues were taken up in 1 ml of *iso*-propyl alcohol and examined chromatographically. In addition to the phenols used as the substrates, a number of other ninhydrin-negative silver-reducing substances was found in each extract. Those that proved to be identical with authentic samples of known phenols are listed in Table 1.

The results shown in this table strongly support the conclusions already reached—that the cuticle of *Schistocerca* is capable of effecting the degradation and decarboxylation of *ortho*-dihydroxyphenols.

## DISCUSSION

In a previous communication (Malek, 1958a) the colourless exocuticle of *Schistocerca* was regarded as more closely homologous with the amber-coloured zone of the exocuticle of *Periplaneta* than with the underlying colourless zone,

TABLE 2—A COMPARISON BETWEEN THE EXOCUTICLES OF *Periplaneta* AND OF *Schistocerca*

| Exocuticle of *Periplaneta* | Exocuticle of *Schistocerca* |
|---|---|
| Amber-coloured | Colourless |
| Refractory to staining | Readily stains |
| Not digested by moulting fluid | Digested by moulting fluid |
| Acid resistant | Not resistant |
| Protocatechuic acid not oxidized | Protocatechuic acid oxidized |
| Hydroquinone oxidized | Hydroquinone not oxidized |
| Hydroxylating system present | Hydroxylating system absent |
| *Para*-quinone tanning | *Ortho*-quinone tanning |

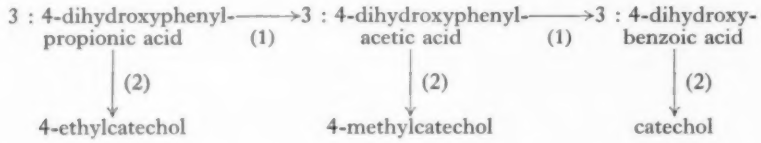
(The information relating to *Periplaneta* is partly taken from Kennaugh, 1958.)

both being phenolically tanned. But in spite of this similarity, there are striking differences in appearance and in structure between the two cuticles (Malek, 1958a). To these already recorded may now be added further differences concerning the nature and metabolism of the phenolic substances involved in their hardening. A summary of the differences between the exocuticles is given in Table 2.

One of the outstanding differences shown in this table is that in place of hydroquinone in *Periplaneta* (Kennaugh, 1958), a number of *ortho*-dihydroxyphenols appear to be involved in hardening the cuticle of *Schistocerca*. These phenols are: catechol, protocatechuic acid, 3 : 4-dihydroxyphenylacetic acid and 3 : 4-dihydroxyphenylpropionic acid. The possibility that other non-aminated diphenols may also participate in exocuticle formation cannot be eliminated. Substituted phenolic compounds of the type 4-methylcatechol are found in *Schistocerca* and are regarded as substrates for polyphenol oxidases (see Richards, 1951). However, none of these *ortho*-diphenols is formed in *Schistocerca* by the non-specific hydroxylating mechanism which gives rise to hydroquinone in the cuticle of *Periplaneta*. Instead, they all seem to be derived from a common substance, 3 : 4-dihydroxyphenylalanine, which may be the phenol formed in the blood by the specific action of tyrosinase on tyrosine.

But although the true nature of the blood phenol is unknown, the immediate parent substance from which are derived all these non-aminated phenols is

3 : 4-dihydroxyphenylpropionic acid. The processes involved are either degradation (1) or decarboxylation (2), and both proceed side by side in the developing cuticle as illustrated below.




It is remarkable that this scheme is fundamentally the same as has already been postulated by Richards (1951). But whether 3 : 4-dihydroxyphenylpropionic acid is directly derived from 3 : 4-dihydroxyphenylalanine, or by way of the lactic derivative (as has been suggested by Richards), remains unknown. Only the lack of an authentic sample of 3 : 4-dihydroxyphenyllactic acid has prevented elucidating this point in the present work. Hackman (1953), on the other hand, has put forward an alternative scheme in which the deamination and degradation of 3 : 4-dihydroxyphenylalanine is not regarded as proceeding through 3 : 4-dihydroxyphenylpropionic acid. It must be emphasized, however, that at that time the occurrence of the latter phenol in an insect cuticle was not known.

The evidence presented in this work seems to leave no doubt that the cuticle of *Schistocerca*, like that of *Periplaneta*, is indeed phenolically tanned as has already been suggested (Malek, 1958a), but that the processes of sclerotization in the two insects follow different routes. In *Schistocerca* sclerotization leads to the formation of an exocuticle which, although hard as in darkly coloured insects, is peculiar in being colourless and chemically unresistant. An explanation of this peculiarity is not immediately clear, but may be sought in the following observations:

(1) The cuticular oxidase of *Schistocerca* is capable of oxidizing protocatechuic acid but not hydroquinone.

(2) None of the tanning phenols, including catechol, is fully oxidized by the cuticular oxidase, all remaining free in the cuticle after it has been fully hardened.

(3) The appearance of substituted phenols during alkaline stannite hydrolysis, as carried out in this work, does not of course necessarily mean regeneration from quinones: if the phenols are present in combination with protein they would still appear, reducing conditions merely protecting them against oxidation in alkali.

These observations may be examined in the light of the views put forward by Mason (1955). According to this author, sclerotization leading to the formation of darkly coloured exocuticles proceeds in two stages. The first stage involves the formation of monosubstituted nuclear derivatives due to reaction between quinones and N-terminal amino groups of protein molecules. The product is an amino-hydroquinone which, in the second stage, becomes oxidized in the presence of excess quinones to the corresponding quinonoid derivative. It seems that if second stage quinone tanning takes place then a cuticle must be coloured, since quinones (both *ortho-* and *para-* types) are themselves coloured. Polymerization and copolymerization which may subsequently take place are probably important

in intensifying the colour. In the light of Mason's interpretations it seems probable that the condensation reactions which take place during hardening may stop in *Schistocerca* at the first stage, when only compounds of the type aminohydroquinone are formed. According to Mason these compounds are colourless, and their formation would therefore leave the cuticle in the colourless condition originally obtaining at the time of moulting. The question now arises: what then prevents these colourless compounds from being oxidized to coloured derivatives, although the tanning phenols are present in excess in the fully formed cuticle? An answer to this question may perhaps be found in the nature of the cuticular oxidase of *Schistocerca*. As already indicated, it is noteworthy that protocatechuic acid is oxidized but hydroquinone is not, in contrast to the state of affairs in other insects that have been examined (Dennell, 1958c; Kennaugh, 1958). In addition, further work, now being carried out, shows that catechol is oxidized by the newly moulted cuticle of *Schistocerca* definitely less vigorously than in darkly coloured insects, so accounting for the persistence of large amounts of unoxidized phenols in the fully hardened cuticles. It may be suggested that it is this failure to oxidize phenols effectively which results in a lack of the *ortho*-quinones needed to oxidize the colourless aminohydroquinones to coloured quinone-tanned proteins, so accounting for the peculiarities of the cuticle of *Schistocerca*.

It is clear from the above discussion that the factor determining the production of colour in a developing insect cuticle may not be so much dependent on the type of the tanning phenols involved as it is on the nature of the cuticular oxidase.

The fact that catechol can be regenerated from fully hardened cuticles of *Schistocerca* is not inconsistent with the above explanation. Mason (1955) regards this phenol, of all the *ortho*-diphenols that have hitherto been identified in insect cuticles, as capable of forming disubstituted nuclear derivatives, and consequently of developing colour in the cuticle. It is to be emphasized, however, that only small amounts of this phenol are present in actual combination with the cuticular protein of *Schistocerca*, so that cross-linking between protein molecules must be small in extent. This would further explain why the exocuticle of *Schistocerca*, in contrast with that of *Periplaneta* (Dennell & Malek, 1956), is readily stained with a variety of dyes (Malek, 1958a) and is digested by the enzymes of the moulting fluid just as readily as it is by concentrated mineral acids (Malek, 1958b).

In conclusion, it is now evident that quite a large number of different phenols combine with the substrate in different ways when the cuticle forms an exocuticle. Indeed, it has already been suggested by Brunet & Kent (1955) and by Wigglesworth (1957) that the process of sclerotization in insects must be of more than one kind. To this must now be added the differing characteristics of cuticular oxidases as a factor in determining the type of exocuticle produced.

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# THORACIC STRETCH RECEPTORS IN CRAYFISH AND ROCKLOBSTER*

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**Abstract**—(1) The properties and functions of thoracic stretch receptor organs have been investigated in *Procambarus clarkii* (Girard) and *Panulirus interruptus* (Randall).

(2) A new terminology is proposed for the specification of the thoracic and the abdominal organs.

(3) It is shown by electrical recording and by staining that each of the first five segmental thoracic nerves is connected to a single receptor cell (N-cell) which is a specialized receptor muscle; the sixth nerve in *Procambarus* innervates a single slow stretch receptor, and in *Panulirus* a fast one in addition.

(4) The seventh and eighth nerves each innervate typical slow and fast receptors which represent the beginning of the abdominal series.

(5) In the crayfish the slow receptors of the sixth nerve and the most posterior N-cell are concerned with detecting abduction of the abdomen on the thorax, those of the two sides being reciprocal; in *Panulirus* the next N-cell has the same function.

(6) The remaining three N-cells in *Panulirus* are located on a slip of the attractor epimeralis muscle and discharge synergistically when the latter is stretched.

(7) In *Procambarus* the most anterior two N-cells have this same location and function; the next two are located on the contractor epimeralis muscle and respond in a manner reciprocal to that of the anterior pair.

(8) It is pointed out that the anterior N-cells may be involved in signalling internal fluid pressures of the thoracic cavity.

(9) The N-cells of *Procambarus* react to acetylcholine with an increase and to gamma aminobutyric acid with a decrease in discharge rate.

## INTRODUCTION

In 1951 Alexandrowicz described in the extensor musculature of the abdomen of *Homarus vulgaris* and *Palinurus vulgaris* structures which he referred to as muscle receptor organs (MRO), postulating that they were sensory and would be activated by stretching as the abdomen was flexed. In each segment a pair of organs occurs on each side, one organ ($RM_1$) being shorter than, and lying lateral to, the other ($RM_2$). Wiersma *et al.* (1953) showed, in *Procambarus clarkii*, that the structures were indeed stretch receptors and discovered that $RM_1$ was a tonic, very slowly-adapting sense organ, while $RM_2$ was more phasic and relatively fast-adapting.

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Meanwhile, Alexandrowicz (1952a) described two pairs of similar organs located in the posterior thorax of *Homarus* and *Palinurus*. These were investigated by Eyzaguirre & Kuffler (1955) who demonstrated that the lateral members of the pairs (Alexandrowicz's MRO VII lat. and MRO VIII lat.) were tonic and comparable with the abdominal $RM_1$, and that the medial members (MRO VII med. and MRO VIII med.) were phasic. These thoracic organs are, therefore, clearly part of the same segmental series as the abdominal organs. Kuffler & Eyzaguirre (1955) made use of the fact that, in contrast to the closeness of the members of a pair of abdominal organs, the medial and lateral organs of the thoracic MRO VIII are more widely separated anatomically. They were thus able to investigate the properties of inhibitory fibers to these organs in *Homarus* and several species of crayfish. Histologically MRO VII med. is unusual in that no inhibitory innervation has yet been found for it.

Further work (Alexandrowicz, 1952b, 1954, 1956) showed that muscle receptor organs also occur in several of the posterior thoracic segments in other higher Crustacea.

Such muscle receptor organs, consisting of a sensory cell connected to a specialized receptor muscle, do not occur in the remaining, more anterior, segments of the thorax in *Homarus* and *Palinurus*, but are present in *Squilla*. For several decapod Crustacea, Alexandrowicz (1952a, 1956) described five nerve cells on the muscles of each side in this region, referring to them as "N-cells" and suggested that they are the homologues of the MRO in the rest of the body. In contrast to the MRO, the N-cells are not associated with differentiated muscle fibers; instead their long dendrites ramify extensively over and between a number of apparently ordinary muscle fibers.

This paper reports on the anatomy and physiology of the N-cells in the crayfish, *Procambarus clarkii* (Girard) and the rocklobster, *Panulirus interruptus* (Randall). An attempt is also made to deduce the functional significance of all the thoracic receptors of these two species.

## METHODS

Different approaches were used to gain access to the nerves of the thorax for recording the impulses arising from these sense organs. The animal was always first decapitated and the stomach, mid-gut gland and gonads removed carefully so as not to expose the nerves and muscles to damaging secretions from these viscera. The claws and legs were cut off close to the body, the branchiostegite cut away over almost its entire free area and the gills removed. Subsequently one of three methods was followed.

1. *The parasagittal preparation.* This was used for most of the experiments and served satisfactorily for general exploratory work as well as for detailed investigations on individual sense organ responses. The eviscerated animal was cut through completely from dorsal to ventral surface a little to one side of the sagittal plane. This operation opens the pericardial cavity and bisects the pericardial floor. Extreme care must be taken at this stage, since distortion of the

pericardial membranes bends the nerves running through them and causes a conduction block. Alexandrowicz (1952a) notes that removal of pericardial tissue leads easily to damage of the cells and axons in question, but we were able to avoid this by careful dissection or by pinning the pericardial floor away from the region being worked on. However, tracing the signals with certainty through that part of the nerve bundles where they traverse the pericardial wall remained the most

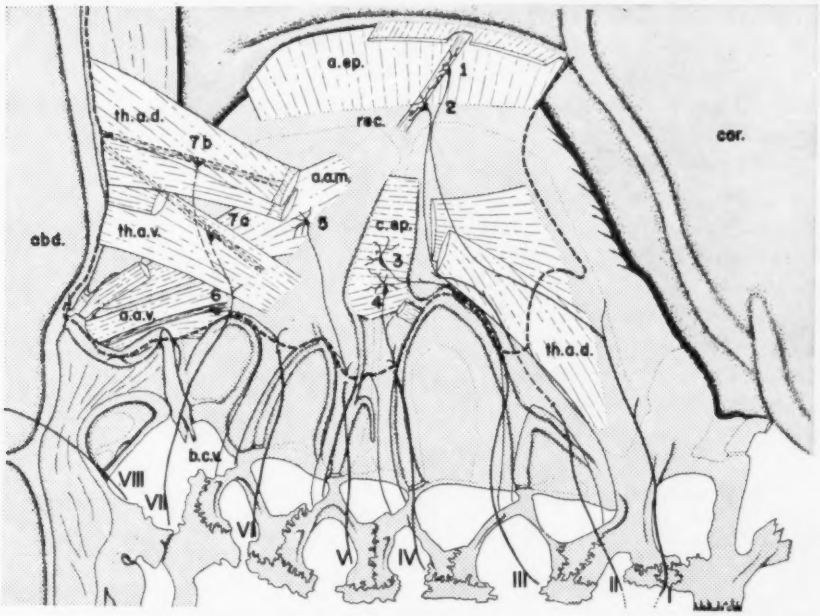


FIG. 1. Semi-diagrammatic drawing of a flattened view of the left side of the thorax of *Procambarus clarkii* (Girard) as seen from the inside of a parasagittal preparation. The locations of the stretch receptors and their innervation are shown. The pericardium is cut away along the thick broken line.

a.a.m., medial head, and a.a.v., ventral head, of abductor abdominis muscle; a.ep., attractor epimeralis; c.ep., contractor epimeralis; rec., receptor slip of attractor epimeralis; th.a.d., dorsal head, and th.a.v., ventral head, of thoraco-abdominal extensor muscle; I–VIII, second roots of thoracic ganglia; 1, SR-$\alpha$; 2, SR-$\beta$; 3, SR-$\gamma$; 4, SR-$\delta$; 5, SR-$\epsilon$; 6, $SR_1$-Th7/8; 7a, $SR_1$-Th/Ab; 7b, $SR_2$-Th/Ab; abd., first segment of abdomen; b.c.v., branchiocardiac vein of the fourth leg; car., carapace.

difficult of the procedures. The heart was removed under a dissecting microscope, and the preparation washed with the appropriate bathing fluid. Long sections of the required nerves could be obtained at this stage by removing the ventrally-located longitudinal flexor musculature; this leaves the main nerves running freely in the body cavity from the cord to near the pericardial floor, where they enter the branchio-cardiac veins (Fig. 1). Further preparation of the nerves was then carried out under guidance of the signals present in them, in order to trace them either towards or from the sense organs.

2. *The ventral preparation.* This was used mainly to investigate bilateral responses of pairs of sense organs, with respect to agonistic or reciprocal functional activity. The thoracic cavity of the eviscerated animal was washed out with bathing fluid and the animal pinned, ventral side up, in a waxed dish. Under a dissecting microscope the limb bases and median parts of the sternites were removed in small pieces. In this way the cord was exposed for short distances. Removal of loose connective tissue and neighboring limb muscles permitted the segmental nerves outside of the pericardial cavity to be prepared symmetrically for recording. This approach has been especially used for the more posterior cells and organs.

3. *Isolated preparations.* These were made variously according to the region under observation. In general they consisted of one pair of sense organs together with the associated muscle(s) and the attached intrapericardial portions of the afferent nerve(s). The calcified skeletal regions to which the muscles attached were held in mechanically manipulated forceps, and in this way steady or varying stretches could be applied. These preparations were made of small circumscribed regions of the thorax and were used to elucidate the circumstances producing responses in specific sense organs. They were also especially suited for the study of the influences of drugs on the sense cells.

The nerve responses were picked up on platinum electrodes, amplified and passed to a dual beam oscilloscope. A speaker was used to monitor nervous activity.

Staining with methylene blue, with or without Rongalit, was carried out on fresh animals and routinely on preparations in which the presence of a sense organ had been determined. By ligating an active nerve in the latter cases before staining, the organ responsible for the response could almost always be readily located.

*Bathing media*

For *Procambarus*, crayfish physiological solution (Van Harreveld, 1936) was used. For the rocklobster we employed at first an artificial medium approximating to an analysis of the blood given by Schlatter (1941), but this proved no better than the more convenient filtered sea-water which was used in the remaining experiments and was quite satisfactory. Experiments were commenced in cold medium which was replaced frequently.

## RESULTS

*Procambarus*

A major difficulty encountered in this work lies in the fact that the nerve trunks containing the pertinent axons give off branches which form into a very complicated plexus on each side of the thorax at the level of the pericardial floor. The sensory axons of the stretch receptor cells (SR) were clearly shown after staining to pass through the plexuses without branching there, but during tracing of these axons while recording from them many false leads had to be followed before the correct pathways were discovered. The plexuses here referred to are no doubt the same as presented difficulties to Alexandrowicz (1932), where he states that in this area the nerves ". . . are interconnected by numerous anastomoses . . ."

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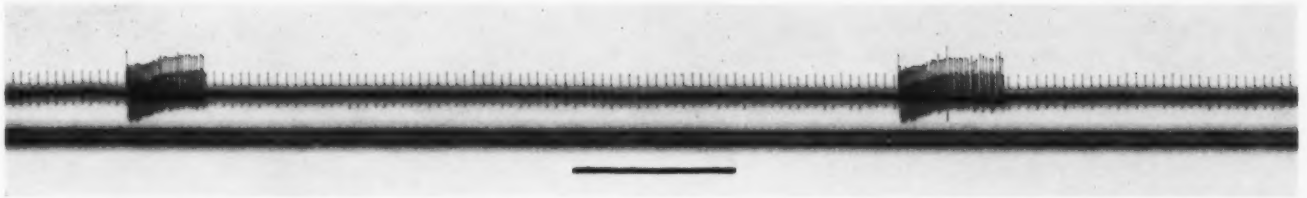

FIG. 2. *Procambarus*—parasagittal preparation. Single lead of SR-$\epsilon$ in main nerve trunk. Cell under slight stimulus producing a discharge of about 17 impulses/sec. Superimposed are two bursts of "spontaneous" activity starting at very high frequency, in which at least two additional fibers appear to be involved. Lower trace, 60 c/s. Time line 1 sec, as in all following figures.

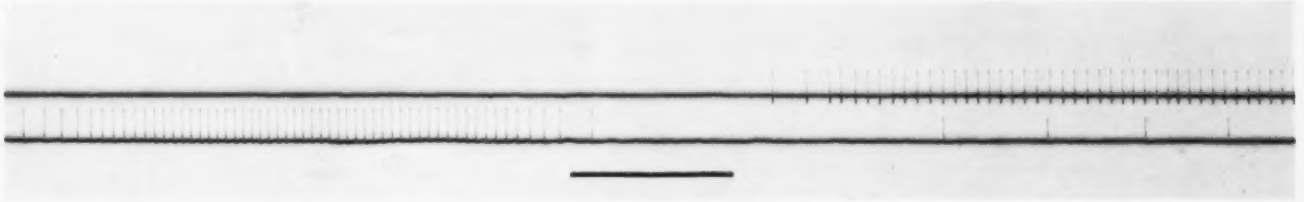

FIG. 3. *Procambarus*—ventral preparation. Simultaneous records of the right (upper) left (lower) nerves of $SR_1$-Th7/8. Initial stimulation by moving abdomen slightly to the right, subsequently slightly to the left. Note tendency of the left cell to exhibit background discharges even when the organ should be relaxed.

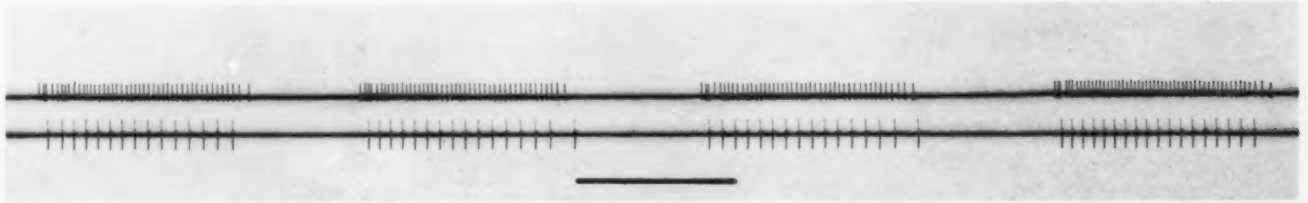

FIG. 4. *Procambarus*—isolated preparation of epimeral contractor muscle. Simultaneous records of SR-$\gamma$ (upper) and SR-$\delta$ (lower). Note the higher discharge frequency in SR-$\gamma$ due to preferential stretch of the upper part of the contractor muscle.

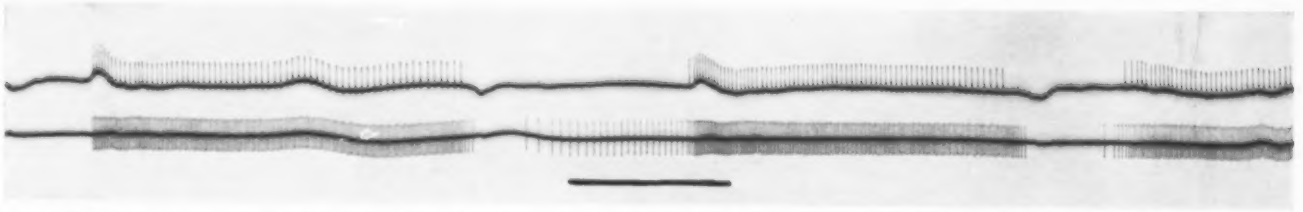

FIG. 5. *Panulirus*—parasagittal preparation. Simultaneous records of SR-$\epsilon$ (upper) and $SR_1$-Th7/8 (lower), of the left side. Note tendency to discharge continuously in the lower trace and the much higher frequency attained. Stimulus: bending the abdomen to the right.

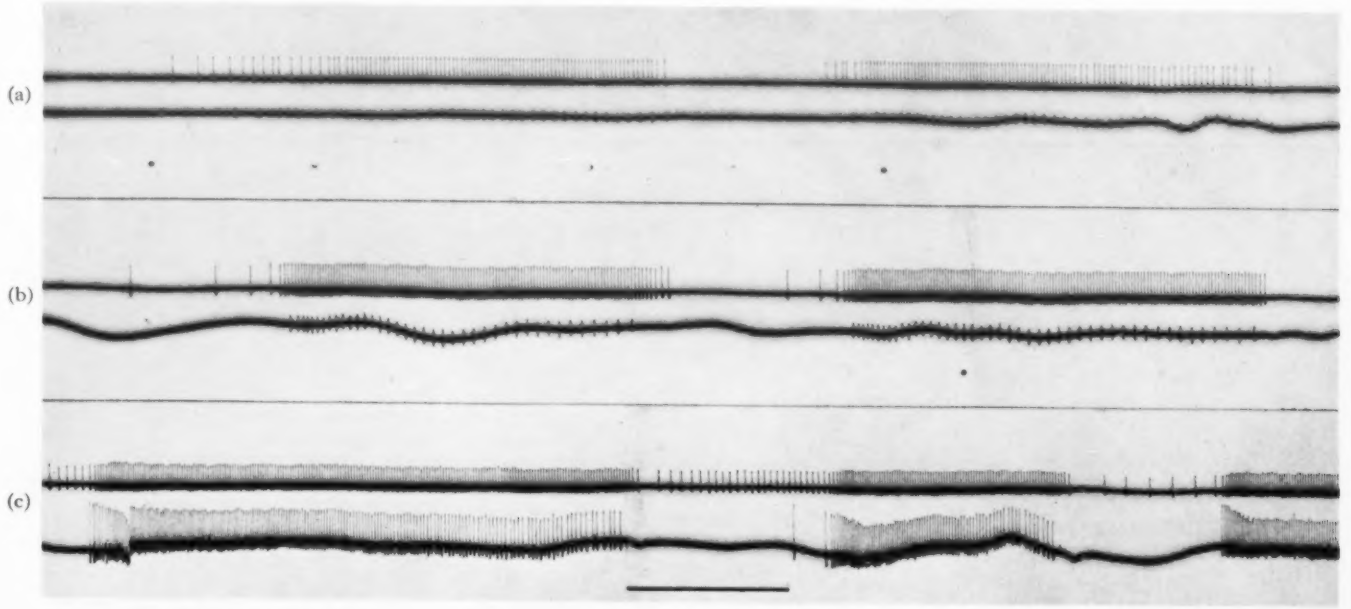


FIG. 6. *Panulirus*—parasagittal preparation. Three records from the same preparation in which the three nerves from SR-$\alpha$, SR-$\beta$ and SR-$\gamma$ were separately prepared. In each case the stimulus was stretching of the receptor slip of the attractor epimeralis muscle. (a) Simultaneous records of SR-$\gamma$ (upper) and SR-$\alpha$ (lower). Note higher frequency and larger size of impulses in SR-$\gamma$. (b) Similar records of SR-$\beta$ (upper) and SR-$\gamma$ (lower). SR-$\beta$ shows a tendency to fire continuously. (c) Similar records of SR-$\beta$ (upper) and SR-$\alpha$ and SR-$\gamma$ combined (lower). The spikes from SR-$\alpha$ are very small. At the commencement of each discharge the frequency of SR-$\gamma$ is higher than that of SR-$\beta$, but the latter cell accommodates more slowly in this preparation.

In recording from the segmental nerve trunks while tracing these to or from the sense organs, we repeatedly obtained nerve impulses which could not be attributed to the sense organs under study. These impulses were either grouped discharges (Fig. 2) or steady low frequency discharges. Their origin, though almost definitely peripheral, has not been elucidated; most likely they arise in the plexuses or possibly in the more medial longitudinal bundles of nerve fibers in the pericardial floor. These bundles are Alexandrowicz's "fasciculi longitudinales" (1932) and are themselves connected to the plexuses. We were unable to determine the functional significance of these discharges (e.g. when rhythmic, they are not connected with any obvious rhythmic movement) and could not influence them by mechanical stimulation. They were completely unrelated to the discharges of the stretch receptor cells but under some circumstances could be mistaken for such. In our early preparations of the most posterior cells such discharges led us temporarily to believe that fast-adapting receptors were present when we obtained a two-fiber discharge after administration of acetylcholine (Pilgrim & Wiersma, 1960).

In many preparations we found that any of the cells under investigation, with the exception of fast-adapting receptors, could show "spontaneous" discharges of low frequency, which could not be stopped except for short periods after a high frequency discharge. However, for every cell we have also encountered cases in which the receptor responded only during appropriate stimulation. Most likely, the "spontaneous" discharges were due to slightly abnormal conditions prevailing during the experiment (see e.g. Fig. 3 [lower] and 6c [upper] for cells with a low frequency discharge under relaxed conditions). Apart from the responses of the receptor organs and the above-mentioned discharges of unknown origin, no centripetal discharges were obtained in nerves I–VII. Thus it appears that the epimeral wall has no other mechanoreceptors, such as hairs.

Because of the increasing difficulty encountered in obtaining good preparations as succeedingly anterior nerves were investigated and because the results are more different from those of the abdominal stretch receptors, the description will proceed from posterior to anterior.

$SR_1$–Th8/Ab 1. The cell body, identical with that of Alexandrowicz's MRO VIII lat. (see Discussion for the reasons of the changed nomenclature), lies with its receptor muscle on the lateral side of the most ventral head of the thoraco-abdominal extensor muscle. The receptor muscle takes its origin together with the extensor muscle on the epimeral plate and is inserted with it on the front margin of the first abdominal tergite.

$SR_2$–Th8/Ab 1. The cell body lies with its receptor muscle on the lateral side of the most dorsal head of the thoraco-abdominal muscle. Its origin is on the most posterior inscription of that muscle head and its insertion is on the first abdominal tergite.

The nerves from these two sense cells run separately for a considerable distance before uniting into a common trunk. The latter then leaves the

pericardium and enters the cord running into the *seventh* thoracic ganglion. The responses from these cells confirmed the results of Eyzaguirre & Kuffler (1955) in that the laterally situated cell ($SR_1$) is slowly-adapting and the medial cell ($SR_2$) fast-adapting. It was further evident that the natural movement of the animal which produces these responses is exclusively flexion of the joint between thorax and abdomen. These cells are therefore functionally equivalent to the various abdominal stretch receptors.

$SR_1$-Th7/8. The cell body and its receptor muscle bundle lie on the medial surface of the most ventral head of the musculus abductor abdominis. This muscle group is referred to by Alexandrowicz (1952a), following Schmidt (1915), as "lateral thoracico-abdominal muscles"; the reasons for the incorrectness of this nomenclature will be presented elsewhere (Pilgrim & Wiersma, in preparation). The axon of this SR runs forward to join a large branch of nerve VI which turns ventrally and leaves the pericardial cavity through the branchio-cardiac vein of the fourth leg to enter the sixth thoracic ganglion. The response of this SR is of the slowly adapting kind and it is clearly comparable with the abdominal $SR_1$ response. The receptor does not, however, respond to flexion of the thoraco-abdominal joint, except for occasional impulses. These are probably due to difficulties in manipulating the bisected animal in such a way as to confine the movement to a single plane. Clearly reproducible responses are obtained by bending the abdomen laterally in that direction which stretches the abdominal abductor of the side on which the cell is located. It thus appears that this sense organ is used for the detection of lateral abduction of the abdomen with respect to the thorax. Excellent confirmation of this hypothesis was obtained by using the ventral preparation in which left and right SR responses were both recorded (Fig. 3). In such preparations the left $SR_1$ responded to bending of the abdomen to the right, the right $SR_1$ to bending to the left. These reciprocal responses are independent of the position of the thoraco-abdominal joint in the vertical plane, i.e. they were obtained equally well at full flexion through to full extension of this joint. It should be pointed out that abduction of the abdomen is quite extensive owing to the remarkable structural anatomy of the hind end of the thorax (further details in Pilgrim & Wiersma, 1960).

$SR_2$-Th7/8. In view of the fact that Alexandrowicz has found such a receptor present in *Homarus* and *Palinurus* and that we had no difficulty in observing it and its reactions in *Panulirus* (see below), it was not until after thorough investigation that we concluded that it is absent in *Procambarus clarkii*. We were never able to record in the appropriate nerves any signal from such a receptor, nor did we succeed in finding such a cell by staining.

*The N-cells*

During the course of this investigation we have been able to find in *Procambarus* the location and function of five peripheral nerve cells, which are undoubtedly the equivalents of the N-cells described for other decapods by

Alexandrowicz. They all conformed to his description in that they are not connected with distinctly differentiated receptor muscle fibers and none receive any fiber which could be interpreted as inhibitory. In these respects they are therefore different from other SR. For all, however, stretch of the muscle fibers on which they are located appears as the natural stimulus, and all five react with long-lasting slowly-adapting discharges to a constant amount of stretch. They thus all resemble $SR_1$ cells in reaction type. Alexandrowicz has variously numbered these cells, but since we have been able to trace them serially, we propose a different nomenclature. From front to back the five cells will be called SR-$\alpha$, $\beta$, $\gamma$, $\delta$ and $\epsilon$. There is a gradual increase in the size of the cell bodies from front to back and this is accompanied by an increase in the size of the signal obtainable from the nerve bundles. There may also be an increase in the maximum frequency with which the cell can fire though it is certain that none of the N-cells reaches the high frequencies at which the abdominal slow stretch receptors can discharge. Instead of a maximum above 200 per sec as for the abdominal $SR_1$, we have rarely encountered any discharge above 100 per sec (see e.g. Fig. 4).

SR-$\epsilon$ lies just within the front edge of the middle head of the abdominal abductor and sends its axon to the fifth thoracic ganglion (i.e. that of the second pereiopod). This muscle head is functionally synergic with the other heads of the abdominal abductor and its action is to bend the abdomen laterally. SR-$\epsilon$ responds to stretch of this muscle head and it fires synergistically with $SR_1$-Th7/8 of the same side. Experiments in which the nerves from these two organs were placed on separate electrodes showed that the two cells responded to the same stimuli, and ventral preparations confirmed that SR-$\epsilon$ of left and right sides are reciprocal in their discharges. The responses can be led off without much difficulty either from the nerve near the cell, the axon running obliquely forward and downwards closely applied to the epimeral wall, or from the main trunk V. In between it joins a nerve trunk which enters the plexus and the signal has to be traced through this region to reveal the axon's course.

SR-$\delta$ *and* SR-$\gamma$. In *Procambarus* these two cells were both found located on the medial surface of the contractor muscle of the epimeral plate. They also resemble each other closely in their reactions, and are therefore described together. However, each enters the central nervous system along a different pathway, namely IV and III (see Fig. 1). Expecting only one cell on this muscle, as in *Homarus*, we were quite surprised in the early stages to find impulses sometimes in trunk IV, sometimes in III from what had to be a receptor on this muscle. Whereas with careful preparation we had no great difficulties later in obtaining a signal in each with regularity, this needed practice. The two receptors are synergistic in their response to stretching the contractor muscle when this was performed on the parasagittal preparation. In such experiments we were able to obtain parallel patterns of discharges in the nerves, but the impulses in the two nerves were quite independent showing that their origins were distinct. The contractor muscle can be made into a satisfactory isolated preparation without much trouble. It was then

possible by stretching either the upper or lower portion preferentially to obtain a greater response either from SR-$\gamma$ (Fig. 4) or from SR-$\delta$. Although the two cells are in general synergic, the possibility of some differential response cannot be excluded for the intact animal. The contractor epimeralis muscle spans a V-shaped thin part of the otherwise calcified epimeral plate and the natural actions of different thoracic muscles might cause different tensions in the upper and lower parts of the contractor muscle. Since the epimeral plate as a whole is naturally bowed inwards along its length, stretching of the muscle will result from any further inpushing of this wall. In contrast any outpushing will slacken the muscle and reduce the discharge of the sense cells. Further discussion of this aspect will be left until the activity of the remaining N-cells is described.

The cell body of SR-$\delta$ lies fairly ventral on the muscle and its axon runs a ventral course joining the main trunk IV below its junction with the plexus. For this reason its signal in the main trunk is the only one remaining after this connexion with the plexus is severed. The cell body of SR-$\gamma$ is situated more dorsad and often slightly anterior of SR-$\delta$. The positions are not constant and at least in some instances the cell bodies lie so close to each other that overlapping of the two sensory fields is a distinct possibility. SR-$\gamma$'s axon runs horizontally for a short distance in front of the contractor muscle and leaves the pericardium through the branchio-cardiac vein of the claw to run into main trunk III of the third ganglion.

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SR-$\beta$ *and* SR-$\alpha$. These proved the most difficult cells to investigate. Largely the problem resolved itself into one of extreme caution in the early preparation of the nerve trunks in which their axons run. Particularly at the places where these trunks penetrate the pericardial membranes was there great danger of kinking the fibers resulting in unresponsive leads. Opening up the pericardial cavity in the parasagittal plane allows the muscles imbedded in the pericardial floor to shorten and so distort the floor grossly. Signals have been obtained from the two nerves II and I quite regularly before the pericard was opened, but because of the thinness of the axons with regard to the diameter of the whole nerves, the signals are weak and, under these circumstances, accompanied by the above-mentioned "spontaneous" discharges. Most of our results have therefore been obtained from the fairly long stretches of the axons within the pericardial cavity but tracings through the wall have also been performed with success for both cells in several instances. Once the course of the fibers had been well established, good preparations could regularly be obtained from the axons at about the middle of their course in the thorax.

Staining showed that the two cell bodies lie well-spaced on the anterior edge of a narrow muscle bundle which runs between the carapace and the epimeral plate inserting on the thin cuticle at the top of the V-shaped thin part, previously mentioned. This bundle is here tentatively regarded as a separate slip of the very extensive attractor epimeralis muscle. Alexandrowicz (1952a) noted that corresponding cells of *Homarus* and *Palinurus* were similarly located on an "inner layer of muscle fibers taking an oblique course" with respect to the neighboring

muscle fibers of the anterior muscle. He did not separately name this slip, nor will we at the present time; but since it is not yet certain that the efferent innervation of these muscle fibers is identical with that of the attractor muscle fibers, the possibility remains that differential contraction or relaxation can bias the responsiveness of these cells. If so, the slip would be a receptor muscle, without showing the histological differentiation such as occurs in the receptor muscle fibers of the organs from SR-Th 7/8 to SR-Ab 6/T.

The axons of the two cells join with other nerve fibers close to the muscle slip, then for a short distance run combined in a common nerve. At about the level of the bottom of the muscle slip, they again separate. By leading off from the nerves at this level a lead can be obtained, with fair ease, in which both signals are present. For leading off a single signal in each channel, further central preparation of both bundles, which is more difficult, is necessary. The nerve branch containing the axon from SR-$\beta$ runs ventrally, close to the epimeral plate passing lateral to the large extensor muscles, and then turns forward to enter II. The branch containing the axon from SR-$\alpha$ passes over the extensor muscles on the medial side, turns forward along these muscles and joins with a larger branch to form nerve trunk I.

As with SR-$\delta$ and SR-$\gamma$, the responses of SR-$\beta$ and SR-$\alpha$ are synergistic but clearly independent in impulse generation. Both parasagittal and isolated preparations gave results which showed that stretching the muscle slip is responsible for their discharges. It was not possible to obtain a differential effect between these two cells since they are, in contrast to SR-$\delta$ and SR-$\gamma$, oriented in series instead of in parallel.

Their function appears to be to register the tension on the muscle slip and thereby indirectly that on the neighboring attractor muscle sheets. It should be pointed out that the latter are all oriented in a nearly horizontal plane, so that stretching will result when the epimeral plate is moved outwards. Thus the natural stimulus is opposite to that which causes discharge in SR-$\delta$ and SR-$\gamma$. These two groups are therefore reciprocal in action. Using a parasagittal preparation with either SR-$\alpha$ or SR-$\beta$ on one lead and with either SR-$\gamma$ or SR-$\delta$ on the other, we were able to show that such reciprocity of action between the two groups does indeed occur, and there is no reason to doubt that this is normal. Using ventral preparations in which the same SR pair was recorded with two leads, we also showed that the left and right homologues acted synergistically. This applied to all the cells from SR-$\delta$ to SR-$\alpha$.

*Action of drugs on the N-cells.* It has already been noted by Kuffler & Edwards (1958) in other species, that the posterior thoracic receptors react to gamma aminobutyric acid (GABA) in the same way as do the abdominal receptors. Particularly interesting is the observation that the response of $SR_1$-Th 7/8 (the cell body of MRO VII lat.), which is not believed to receive an inhibitory fiber, is nevertheless inhibited by GABA.

We have applied ACh and GABA to all N-cells of *Procambarus* and found that they all reacted to these substances. ACh applied to the cells caused discharges in previously silent cells, or an increase in frequency in cells already firing. The

threshold concentration for the action of ACh was about $10^{-6}$ wt/vol and with higher concentrations the frequency of discharge further increased, but never reached the high levels of the abdominal stretch receptors. Overstimulation with ACh has been noted but at frequencies well below those for abdominal $SR_1$.

GABA caused reduction or cessation of the accommodated discharge of the cells when applied in concentrations of $10^{-5}$ wt/vol or greater. At lower concentrations the effect was often present though less marked. The N-cells thus resemble the other stretch receptors closely with respect to these pharmacological actions.

*Panulirus*

The investigations on this species have been less extensive, since only a limited number of animals was available. As a result we have not used the ventral preparation at all, the experiments being limited to parasagittal and isolated ones. We have, nevertheless, confidence in the results obtained since the interpretation of the experimental data was facilitated by the experience gained with *Procambarus*, and the cell locations did not materially differ from those described by Alexandrowicz for *Palinurus*.

As in *Procambarus*, the posterior thoracic segments contain sense organs with specialized receptor muscle bundles, while the anterior sense organs are five N-cells. The axons from all the stretch receptors in the thorax of *Panulirus* run by the segmental nerve trunks to ganglia in the same unbroken sequence, so that it appears justified to homologize the cells in this animal with those innervated by the same ganglia in *Procambarus*. They will therefore be named correspondingly, notwithstanding the differences in the peripheral locations.

Physiologically there was also considerable similarity between the same cells of the two species, in that the maximum frequency of the discharge of the N-cells was well below that of the abdominal stretch receptors. Furthermore, there was again a decrease with respect to height of signal and to cell-size as more anterior N-cells were investigated, which was especially clear when SR-$\epsilon$ and SR-$\delta$ are compared with the three anterior cells. Most attention has been focused on the N-cells, but some significant observations were also made on the stretch receptors of the posterior thorax.

$SR_1$-Th8/Ab 1 *and* $SR_2$-Th8/Ab 1. The responses of these two cells were readily obtained in the seventh nerve and the signals were very large. The natural stimulus is undoubtedly flexion of the thoraco-abdominal joint. The location and histology of these receptors were those expected by comparison with other species.

$SR_1$-Th7/8. In nerve VI a large slowly-adapting response was obtained following abduction of the abdomen in such a way as to stretch the side under examination (Fig. 5). The organ responsible for this discharge consists of a cell on a definite receptor muscle located in the most ventral head of the abductor abdominis. Its position conforms to that described by Alexandrowicz for *Palinurus* (1952a) and to that in *Procambarus*.

$SR_2$-Th7/8. In contrast to the findings in *Procambarus*, a strong signal was readily obtained in addition to the one from the above described organ in the same nerve trunk. This phasic discharge was produced by movements of the abdomen on the thorax in several ways, but in the parasagittal preparations we were unable to make certain which plane of movement should be considered as the natural one. The receptor muscle lies, however, as described by Alexandrowicz (1952a) attached between the two inscriptions of the most dorsal head of the thoraco-abdominal muscle. It lies on the lateral side of this muscle and appears to be more superficial than in the other species. This location means that it lies in series with $SR_2$-Th 8/Ab 1 and will thus function synergistically with this receptor.

*N-cells.* A major difference between the crayfish and the rocklobster, in this respect, is the absence of a contractor epimeralis muscle in the latter.

SR-$\epsilon$. This cell was found to lie on the medial surface of the middle head of the abdominal abductor muscle, at one-fourth to one-third from the anterior end, thus more posterior but on the same muscle as its location in *Procambarus*. Its response, like that of $SR_1$-Th7/8 was elicited by lateral bending of the abdomen (Fig. 5).

SR-$\delta$. Its cell body lies on the same muscle head as the previous one but nearer to the anterior end shallowly imbedded between the superficial muscle fibers. Its activity parallels that of SR-$\epsilon$. It therefore appears that abduction of the abdomen is registered here by the three organs: SR-$\delta$, SR-$\epsilon$ and $SR_1$-Th7/8 simultaneously. It may be significant in this connexion that in *Panulirus* the angular displacement possible is greater than in the crayfish, in which only two receptors signal this movement. The three SR of the two sides will undoubtedly act reciprocally as those of *Procambarus* do. It was interesting to note that the course of the axon of SR-$\delta$ is quite complicated. It runs at first horizontally forward until it enters a plexus, as revealed by staining. From here on physiological tracing showed that it leaves this region to continue in the fourth nerve which, however, joins the plexus at some distance from where the axon enters.

SR-$\gamma$. Like SR-$\delta$ its cell body shows a different location from that in *Procambarus*, namely on a receptor slip of the epimeral attractor muscle on which the two anterior cells are also located. Its axon leaves this slip in a posterior direction before turning ventrally, to join the third nerve. Whereas in *Palinurus* the cell body is presumably the most ventral one on the receptor slip, we found that in *Panulirus* its position is somewhat variable but very near that of SR-$\beta$. These two cells may have thus considerably overlapping dendritic fields.

SR-$\beta$. The cell body is located about mid-length and usually close to or even in front of the anterior margin of the receptor slip. Its axon runs first anteriorly for a short distance before turning ventrally to join the second nerve trunk.

SR-$\alpha$. The cell body lies about one-third from the dorsal origin of the slip on the anterior aspect. Its axon runs almost horizontally forward, laterad of one of the heads of the attractor muscle and never joins in a common nerve with the axon from SR-$\beta$, in contrast to the situation in the crayfish.

Whereas the signals of these cells in the main trunks of the ventral thorax were very small and could only just be used for tracing purposes, it was possible to obtain satisfactory ones from the nerves near the receptor slip. From this region leads could be obtained for all three cells of one preparation. Examination of such records (Fig. 6 a, b, c) left no doubt that the cells fire independently. It was not possible to activate any one cell preferentially by stretch of the muscle slip as a whole.

## DISCUSSION

Because of considerable ambiguity which has arisen in the nomenclature with regard to several aspects of anatomy concerning the stretch receptors, we propose a new terminology which includes all the abdominal and thoracic stretch receptors. It was shown in *Procambarus* (Hughes & Wiersma, 1960) that the axon of each abdominal stretch receptor enters the abdominal ganglion lying in the segment anterior to that containing the sense cell. This has subsequently been shown for other Decapoda as well (Pilgrim, 1960) so that it may be considered a general phenomenon. This led us to doubt the validity of Alexandrowicz's assignment of the names thoracic VIII and VII to the posterior thoracic receptors, if this is read to mean that they enter the correspondingly numbered ganglia. In the present investigation we therefore followed these nerve fibers with great care and found that indeed in the thorax as in the abdomen there exists a backward slant of the neurotome with respect to the dorsal myotome. Thus $SR_1$- and $SR_2$-Ab 1/2 were found to enter the eighth thoracic ganglion, and from here forward successive axons always entered one ganglion more anterior. Consequently, SR-$\alpha$ must be considered as belonging to the first thoracic ganglion, which ventrally innervates the first maxilliped. Because all abdominal SR run in second roots, we believe that all the lateral main nerve trunks I-VIII described in this paper must be considered to be such roots. However, the place where the roots leave the cord becomes more and more dorsal from the eighth thoracic ganglion forward, so that those dorsal roots of the subesophageal ganglion which Keim (1915) designates $ns_2$–$ns_4$ must be considered the second roots of the first three thoracic segments.

The incorrect assignment of the nerves of the posterior part of the thorax to the ganglia may have come about through the peculiarities of the second roots in this region. Those of the seventh and eighth ganglia approach the perineurium of the cord closely, but do not enter at that point. Instead the nerve trunks run forward in their own sheath which is only loosely connected to the cord by the same connective tissue which attaches the blood vessels. The most misleading in this respect is the second root of the seventh ganglion, which appears to belong to the eighth ganglion but in fact does not enter the cord tissue until it reaches the back of the seventh. Similarly the second root of the sixth ganglion attaches to the cord only just in front of the seventh and runs along the long connective flanking the sternal artery.

Our reasons for proposing a new terminology are based on these considerations. The term SR (= stretch receptor) as used in this paper applies to the entire

receptor cell including its dendrites, cell body and axon (peripheral and central parts). This term is more specific than SN ( = sensory neuron) which was used by Florey & Florey (1955) for the abdominal SR, since their term does not provide a distinction between these and other sensory neurons. The SR are further specified according to their reaction type and to their location. For example, $SR_1$-Ab 3/4 is the slowly-adapting receptor which signals flexion of the fourth abdominal segment on the third. It enters the cord by the second root of the second abdominal ganglion. Previously this receptor was called $RM_1$ of the fourth abdominal segment by Wiersma (1958). The main thoracic nerve trunks formed by the second roots are here numbered after their ganglia of origin. The advantage of the terminology proposed is that it will be readily applicable to more primitive forms in which thoracic segments retain their mobility; it is thus useful for comparative purposes.

In our view there can be little doubt that all N-cells are derived originally from $SR_1$-type cells with a receptor muscle. The regularity of the segmental innervation, the absence of any case in which a N-cell occurs together with a $SR_1$ in the same segment, and the presence of a N-cell and a $SR_2$ in several segments of *Leander* (Alexandrowicz, 1956) strongly support this view. Note, however, that at present the N-cells in *Leander* cannot be homologized with those of the other decapods, since it appears that the most posterior of them does not correspond to our SR-$\epsilon$, but either to $SR_1$-Th7/8 or even to the next posterior. Alexandrowicz (1956) has offered the two alternative suggestions that N-cells are either primitive or degenerate receptors. From our findings we conclude that at least in the higher Crustacea they must be considered as degenerative $SR_1$. Evidence indicates that the $SR_2$-type receptors are more liable to be completely suppressed, as shown by the reduction in number in the series: *Squilla-Leander-Panulirus-Procambarus*. In three species of crabs (*Cancer anthonyi*, *C. antennarius*, *Loxorhynchus grandis*) we were unable to detect either type.

For the view that N-cells are degenerative $SR_1$ pleads also the variability of their locations in single species and the change in muscle with which they associate in different species. *Homarus*, *Procambarus* and the rocklobsters all differ in this respect. Because of this shifting of cells from one muscle system to another, their function is also considerably different between species, especially in the case of SR-$\gamma$ and SR-$\delta$. There is thus a strong impression that this type of cell is available for whatever function is most appropriate in a particular species as the thoracic segments become fused.

Functionally the anterior N-cells which react to displacement of the epimeral plate with respect to the carapace might be involved in signalling pressure changes within the thoracic cavity. Under the conditions of our experiments we were unable to demonstrate such an effect. In no case have we observed that the heart beat caused discharges in ventral preparations, but because of the unavoidability of opening main blood vessels, this is not unexpected. More significant was our failure to induce responses in a ventral preparation in which fluid was injected into the intact pericardium, since a brief but finite pressure increase was induced.

However, because of the removal of the gut, the ventral wall of the pericardium probably absorbed most of the tension. It is therefore still quite possible that in the intact animal fluid pressure changes are registered by these sense cells.

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# THE COMPOSITION OF THE ENDOLYMPH, PERILYMPH AND OTHER BODY FLUIDS OF ELASMOBRANCHS

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**Abstract**—(1) Serum, cranial fluid, perilymph, endolymph and jelly from the ampullae of Lorenzini of *Raja* have been analysed for Na, K, Cl and urea.

(2) The concentrations in mM/kg water are:

| | Na | K | Cl | Urea |
|---|---|---|---|---|
| Perilymph | 281 | 3·54 | 321 | 447 |
| Endolymph | 295 | 63·4 | 391 | 381 |
| Lorenzini jelly | 443 | 12·5 | 581 | 74·8 |

Serum and cranial fluid are similar to perilymph.

(3) The endolymph has 19 times as much K as the perilymph.

(4) The composition of the Lorenzini jelly is unlike that of any other fluid.

(5) Freezing-point depressions show that Lorenzini jelly is 5 per cent hypertonic to sea water, and the other fluids are all approximately 2 per cent hypertonic.

## INTRODUCTION

It is well established that the endolymph of the mammalian ear contains a very high concentration of potassium, about 30 times as much as in the perilymph, the sodium being less concentrated (Smith *et al.*, 1954; Citron *et al.*, 1956; Rauch & Köstlin, 1958). The functional significance of the high potassium concentration is not known, nor is its relationship with the cochlear and vestibular d.c. potentials (see Davis, 1957). On the other hand, in *Octopus* it has recently been shown that the analogous fluids are essentially similar to each other and to the blood (Amoore *et al.*, 1959).

The two previous reports on the composition of the labyrinthine fluids of elasmobranch fishes both indicate differences in potassium concentration between perilymph and endolymph of less than a factor of two (Kaieda, 1930; Jensen & Vilstrup, 1954); but neither are convincing. Kaieda reported, for example, that the endolymph as a whole was twice as concentrated as the perilymph, and the value for perilymph potassium given by Jensen & Vilstrup is much higher than one would expect from the earlier estimates of plasma potassium (Smith, 1929; Hartman *et al.*, 1941). The results of all these determinations are summarized in Table 1.

We have therefore reinvestigated the sodium, potassium and chloride contents of the two ear fluids in rays, and have also estimated the urea because of its importance in the maintenance of osmotic equilibrium in these fishes (Smith, 1929). For comparison, samples of blood serum and of the fluid from the cranial

TABLE 1—ANALYSIS OF LABYRINTHINE AND OTHER FLUIDS: RESULTS OF PREVIOUS INVESTIGATIONS

| Species | Fluid | Na | K | Cl | Urea |
|---|---|---|---|---|---|
| Man[1] | Perilymph | 135–150 | 7–8 | 135 | |
| | Endolymph | 13–16 | 140–160 | 120–130 | |
| Guinea-pig[2] | Perilymph | 150 | 4·8 | 121·5 | |
| | Spinal fluid | 152 | 4·2 | 122 | |
| | Endolymph | 16 | 144 | 107 | |
| *Octopus vulgaris*[3] | "Perilymph" | 555 | 17 | | |
| | "Endolymph" | 601 | 20 | | |
| | Blood | 525 | 30 | | |
| *Scolioidontus laticandus*[4] | Perilymph | 232 | 14 | 248 | 250 |
| | Endolymph | 550 | 24 | 469 | 410 |
| *Acanthias vulgaris*[5] | Perilymph | 247 | 37 | | |
| | Endolymph | 280 | 60 | | |
| *Raja erinacea*[6] | Plasma | 254 | 8 | 255 | 320 |
| *Raja stabuliformis*[7] | Plasma | 255 | 4·9 | 240 | 453 |
| | C.s.f. | 264 | 4·1 | 263 | 436 |

These values are in mM/l; the *R. stabuliformis* were taken from sea water with 17‰ chloride.

References: [1] Rauch & Köstlin (1958); [2] Smith *et al.* (1954); [3] Amoore *et al.* (1959); [4] Kaieda (1930); [5] Jensen & Vilstrup (1954); [6] Hartman *et al.* (1941); [7] Smith (1929).

cavity were also taken, and freezing-point depressions were measured. The cranial fluid is no doubt homologous with the cerebrospinal fluid of mammals, although it contains a clotting protein, and there is no dura mater in fish.

The composition of the jelly which fills the tubes of the ampullae of Lorenzini was also studied and its electrical conductivity found, in connexion with the investigation by one of us of the function of these sense organs (Murray, 1960b).

## MATERIAL AND METHODS

Samples for analysis were obtained from twenty-seven fish, nearly all *Raja clavata*, but a few individuals were of other species (see Appendix). The fish were trawled from sea water of salinity 35·3‰ (Marine Biological Association's records) and were then transferred to aquarium tanks for up to several days. The salinity of the tank water for fish 19–30 was 33·2‰ (calculated from $\Delta = 1{\cdot}81°$, see Knudsen, 1903). Unfortunately, the exact salinity of the tank water from which fish 1–18 were taken is not known because we did not realize that there were two separate sea-water circulations in the laboratory. However, the salinity in the latter case was near to or if anything slightly lower than 33·2‰

(M.B.A. records) and there was, for example, no significant difference between the mean concentrations of sodium in the cranial fluids of the two groups of fish.

Only healthy fish were used, and they were killed immediately before the samples were taken. Colourless blood serum was obtained by bleeding the fish from the exposed heart and allowing the blood to clot and stand for 2–3 hr. The cranial fluid was readily obtained by pipette from the cavity of the skull in front of the brain, and the perilymph was pipetted out in 0·5–1 ml amounts from each ear. For endolymph samples about 0·5 g of jelly could be obtained from each sacculus, and small amounts of fluid (0·01–0·1 g) were pressed out of the semicircular canals and their ampullae after drying the outside on filterpaper to avoid contamination by perilymph. The sacculus jelly is insoluble in water and so was broken up by being sucked into and out of a fine pipette, before being allowed to stand with about 100 times its own volume of water for the salts to diffuse out.

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The jelly from the ampullae of Lorenzini was obtained by squeezing from the long tubes which run on the dorsal surface from the hyomandibular capsule to the antero-lateral and posterior margins of the wing. In some of the samples the first squeeze, i.e. the jelly from the distal part of the tubes, was analysed separately from the jelly from the proximal part. The jelly was dissolved in warm distilled water.

Sodium and potassium were estimated with an EEL flame photometer, chloride by Conway's microdiffusion method (Conway, 1947), and urea by hydrolysis with urease and colorimetric estimation using Nessler's reagent and an EEL photo-electric absorptiometer. Conductivities were measured by passing a known current along a $5 \times 5$ mm perspex trough containing the sample, and measuring the potential difference between two intermediate Ag/AgCl wire electrodes 4 cm apart with a Pye pH meter used as a high-impedance valve voltmeter.

In the experiments on fish 19–30 the freezing-point depressions of cranial fluid, perilymph, endolymph and Lorenzini jelly were measured by the method of Ramsay & Brown (1955), and in these fish only the sodium and potassium in the cranial fluid were assayed.

There are two different kinds of comparison to which the results may be subjected. The first is between our results and those of other workers on other animals. For this comparison each mean value has a standard error calculated for it, and thus allowance can be made for the considerable variation between individual fish. But most of the comparisons which have been made here are between the different fluids from the same fish; suitable statistical analysis (e.g. analysis of variance) can discount that part of the total variation made up by the differences between the individuals, and so permits more accurate comparisons to be made between the different fluids. Because not every fluid was analysed in every fish, the comparisons between the fluids are based only on the fish in which all the relevant fluids have been analysed. The complete individual results are given in the Appendix.

The results are expressed as mM/kg water, which allows direct osmotic comparisons to be made. The water contents found by drying at 105°C for 24 hr were (% w/w): serum, 92·0; cranial fluid, 94·5; perilymph, 94·7; endolymph jelly, 94·7; Lorenzini jelly, 94·8.

The subscripts SE, CR, PE, EN and LO are used to refer to serum, cranial fluid, perilymph, endolymph and Lorenzini jelly, respectively. U is used to refer to urea.

## RESULTS

In six fish the analysis was complete for sodium, potassium, chloride and urea, from serum, cranial fluid, perilymph, endolymph and Lorenzini jelly. But fish 14 was of a different species (*R. montagui*) from the rest (*R. clavata*) and some of the absolute values obtained from it, especially for potassium, lie outside

TABLE 2—ANALYSIS OF LABYRINTHINE AND OTHER FLUIDS OF *R. clavata*: MEANS AND STANDARD ERRORS FOR FISH 9, 10, 11, 13 AND 16 (mM/kg WATER)

| | Serum | Cranial fluid | Perilymph | Endolymph jelly | Lorenzini jelly |
|---|---|---|---|---|---|
| Sodium | 289 ± 4·9 | 280 ± 5·7 | 281 ± 6·9 | 295 ± 5·2 | 443 ± 9·3 |
| Potassium | 3·98 ± 0·20 | 3·34 ± 0·16 | 3·54 ± 0·23 | 63·4 ± 1·9 | 12·5 ± 0·36 |
| Chloride | 311 ± 10·3 | 311 ± 10·2 | 321 ± 10·7 | 391 ± 5·8 | 581 ± 11·6 |
| Urea | 444 ± 12·4 | 437 ± 15·6 | 447 ± 12·5 | 381 ± 8·1 | 74·8 ± 10·2 |
| Na + K + Cl | 604 | 594 | 608 | 749 | 1037 |
| Cl − (Na + K) | 18 | 28 | 34 | 33 | 125 |
| Na + K + Cl + U | 1048 | 1031 | 1055 | 1130 | 1112 |

the range of variation of the other five, although the differences between the fluids are similar. The mean values for the five *R. clavata* are given in Table 2 (when two separate samples were taken of the Lorenzini jelly, their mean has been used here).

There are no significant differences between serum, cranial fluid and perilymph for any of the substances, except that $[K]_{SE}$ is slightly high ($P < 0{\cdot}05$). This may be due to leakage from the red blood corpuscles, although it is said to be only slight in the dogfish (Hempling, 1952). The three fluids are also isotonic, and are all slightly hypertonic to the water in the tank ($\Delta_{water} = 1{\cdot}81°$; $\Delta_{CR} = 1{\cdot}84 \pm 0{\cdot}003°$; $\Delta_{PE} = 1{\cdot}85 \pm 0{\cdot}016°$; $\Delta_{SE} = 1{\cdot}85°$, two fish only).

In comparison with these three fluids, the endolymph is different in all four substances. $[Na]_{EN}$ is slightly but significantly higher ($P < 0{\cdot}05$) than $[Na]_{PE}$, and $[K]_{EN}$ is 18 times greater than $[K]_{PE}$; both these results are confirmed by the values obtained from the other fishes (Table 3). If the combined results of all the fishes are used, $[K]_{EN}$ is 19 times $[K]_{PE}$. $[Cl]_{EN}$ is evidently higher, and $[U]_{EN}$ lower than the values of [Cl] and [U] in the other three fluids. But the cation deficit of measured ions in the labyrinthine fluids is the same, as $[Cl]_{EN}$ is greater

than $[Cl]_{PE}$ by the same amount that $[Na+K]_{EN}$ is greater than $[Na+K]_{PE}$. In total electrolytes, $[Na+K+Cl]_{EN}$ is 141 mM/kg water greater than $[Na+K+Cl]_{PE}$, but because $[U]_{EN}$ is 65 mM lower than $[U]_{PE}$, the totals including urea are only 75 mM/kg water different. The assayed substances are more concentrated in the endolymph than in the perilymph, but the measurements of the freezing-point depression show that the osmotic pressures are not significantly different ($\Delta_{EN} = 1{\cdot}86 \pm 0{\cdot}011°$; $\Delta_{PE} = 1{\cdot}84 \pm 0{\cdot}016°$).

TABLE 3—ANALYSIS OF LABYRINTHINE FLUIDS: MEANS AND STANDARD ERRORS FOR FISH 1, 3, 6, 7, 8, 12, 14 AND 15 (mM/kg WATER)

| | Perilymph | Endolymph |
|---|---|---|
| Sodium | 270 ± 3·7 | 286 ± 8·7 |
| Potassium | 3·22 ± 0·19 | 62·8 ± 1·4 |

As it may be argued that the sacculus jelly is not characteristic of the endolymph as a whole, the Na:K ratio of the sacculus jelly was compared with that of the fluid from the semicircular canals. Because of technical difficulties, evaporation from the small fluid samples was not prevented, and so absolute values for sodium and potassium were not obtained. For the five fish from which

TABLE 4—ANALYSIS OF THE LORENZINI JELLY: MEANS OF FIRST AND SECOND SQUEEZES FROM FISH 10, 11, 14, 16, 17 AND 18 (mM/kg WATER)

| | First | Second |
|---|---|---|
| Sodium | 415 | 419 |
| Potassium | 12·1 | 13·5 |
| Chloride | 562 | 562 |
| Urea | 61·5 | 93·6 |
| Na + K + Cl | 989 | 994 |
| Na + K + Cl + U | 1050 | 1088 |

fluid endolymph samples were taken, the mean Na:K ratios were 4·74 for fluid and 4·62 for jelly. These two ratios are not significantly different ($P > 0{\cdot}05$). Moreover, the two samples of endolymph jelly tested had the same freezing-point depression as the fluid endolymph.

The Lorenzini jelly has quite a different composition, and there are some differences between the contents of the proximal and distal ends of the tubes; these are summarized in Table 4. The stiffness, too, varies, for the jelly squeezed out first from the distal ends of the tubes is stiffer than the jelly from the proximal ends, although no difference in water content was found. The urea from the second squeeze is clearly more concentrated and the difference in potassium is

also significant ($P < 0{\cdot}01$). The sodium and the chloride are similar. Both the differences are in the direction which would result from the diffusion into the sea water at the open end of the tubes of the high potassium and urea concentrations secreted at the proximal end. The jelly as a whole is about 5 per cent hypertonic to the sea water ($\Delta_{LO} = 1{\cdot}91 \pm 0{\cdot}014°$).

The conductivities of the cranial fluid and Lorenzini jelly are given in Table 5. As a check, the conductivity was also determined of artificial solutions made up according to our analysis of those fish on which the conductivities were measured. Because the analysis shows a cation deficit, in making these artificial solutions

TABLE 5—CONDUCTIVITY (AT 20°C) OF CRANIAL FLUID AND LORENZINI JELLY: FISH 10, 11, 13, 14 AND 16

| | Cranial fluid | Lorenzini jelly |
|---|---|---|
| Measured conductivity (mhos) | $0{\cdot}021 \pm 0{\cdot}0011$ | $0{\cdot}040 \pm 0{\cdot}00054$ |
| Conductivity of artificial solutions | 0·023 | 0·043 |

The conductivity of sea water of 33·2‰ salinity = 0·046 mhos (Thomas *et al.*, 1934). The artificial solutions should have approximately 5 per cent higher conductivities than the natural because of the greater dry weight of the latter.

extra cations have to be added in order to be able to achieve the necessary chloride concentration. In this case equal parts of calcium and magnesium were used, so the two artificial solutions had the correct sodium, potassium, chloride and urea contents but had 11 and 38 mM/kg water of calcium + magnesium respectively. The artificial solutions had approximately the same conductivities as the corresponding natural solutions when allowance was made for the differences in water content.

Freezing-point depressions were also determined for artificial solutions corresponding to perilymph, endolymph and Lorenzini jelly. In these solutions the extra cation added was magnesium. The values of $\Delta$ for these artificial solutions were: perilymph, 1·84°; endolymph, 1·98°; Lorenzini jelly, 2·04°.

## DISCUSSION

It is clear from these results that there is a much higher concentration of potassium in the endolymph than in the perilymph or in any of the other body fluids sampled. In this respect, therefore, the elasmobranch ear is similar to the mammalian, although $[K]_{EN}$: $[K]_{PE}$ is approximately nineteen compared with thirty in the mammal. The similarity does not extend to the other electrolytes, for mammalian endolymph has a low sodium content, the chloride being nearly the same as in the perilymph. By contrast, in the elasmobranch $[Na]_{EN}$ is significantly higher than $[Na]_{PE}$ and so with the extra potassium there is a higher concentration of the two cations in the endolymph than in the perilymph. This greater cation

concentration is balanced by a higher concentration of chloride, so that the excess of chloride over sodium + potassium remains the same.

The results obtained here are in reasonable agreement with the sodium values found by Jensen & Vilstrup, for they also found more sodium in the endolymph than in the perilymph. The endolymph potassium agrees as well, but their figure for perilymph potassium would seem to be exactly 10 times too high. Kaieda's results are difficult to interpret as, for example, he reported that the sodium, potassium, chloride and urea were all approximately twice as concentrated in the endolymph as in the perilymph, and there is little agreement with any of our results.

The ionic differences between endolymph and perilymph are accompanied in both elasmobranch and mammal by a potential difference (endolymph positive). In the guinea-pig the potential difference in the cochlea is 50–80 mV (Békésy, 1952) and in the semicircular canals 50 mV (Trincker, 1957), although the utricular potential is reported as only 5 mV (Tasaki, 1957). In the mammal, the potassium must be concentrated against both electrical and chemical gradients (it is tempting to suppose that the potential originates from the active transport of the potassium) and the chloride must be held level against the electrical gradient, whereas the sodium may be in electrochemical equilibrium. But it is possible that not even the sodium is in equilibrium since according to the Nernst relationship a 50–80 mV potential difference corresponds to a 7–24 times concentration ratio, and the published concentration ratios for sodium range from 2·5 to 9 times in the cat and from 6·5 to 9 times in the guinea-pig (Table 1).

In the elasmobranchs also, preliminary experiments with *Scyliorhinus canicula* (Lowenstein, personal communication) indicate that there is a potential difference between the inside of the sacculus and the body tissues outside, but that it is certainly less than 10 mV and probably less than 5 mV (endolymph positive). Here neither the sodium nor the potassium can be in electrochemical equilibrium, but our measured differences in chloride would be balanced by a potential difference of 6 mV.

To a first approximation, the concentration of urea in the various fluids makes up for the osmotic difference between the total of electrolytes and the external medium, as suggested by Smith (1929). In fact, the body fluids (fish 19–30) were about 2 per cent hypertonic to the tank water. This means that the fish had regulated to the lower salinity of the tanks before being killed.

Comparison between the freezing-point depressions of the fluids and their artificial counterparts affords a check on the analysis and gives some evidence concerning the other substances which were not estimated. First, although the artificial perilymph was very nearly isotonic with the natural fluid ($\Delta_{PE} = 1{\cdot}85°$; $\Delta_{ART.PE} = 1{\cdot}84°$), there are other points to consider: published estimates for calcium and magnesium in plasma are about half that used in the artificial perilymph (Smith, 1929; Hartman *et al.*, 1941), and these concentrations, together with the 3 mM/l carbonate (Smith, 1929) would still leave a considerable cation deficit. However, it is known that trimethylamine oxide (TMO), which is a

weak base, is present in body fluids of elasmobranchs at concentrations of 30–100 mM/l (Smith, 1936), and this, rather than magnesium, may account for the deficit in our calculation.

The endolymph contains about 8 per cent more assayed substances than the perilymph, and the artificial endolymph has a freezing-point depression 8 per cent greater than that of the artificial perilymph. But as the actual endolymph is virtually isotonic with the actual perilymph ($\Delta_{EN} = 1{\cdot}86°$; $\Delta_{PE} = 1{\cdot}85°$) there must be considerable differences in un-assayed substances between endolymph and perilymph. The cation deficit cannot be made good with only calcium and magnesium. Perhaps the presence of TMO, proteins, hyaluronic acid (Vilstrup *et al.*, 1953) or other organic substances, by reducing the activity of the assayed ions, could account for the isotonicity of the endolymph.

The composition of the jelly in the ampullae of Lorenzini is unlike that of any of the other fluids. In so far as it is the fluid secreted by a sense organ of the acoustico-lateralis system, it may be homologous with the endolymph, and thus the accumulation of potassium (4 times the serum concentration and 120 per cent of sea water) may be homologous with the accumulation of potassium in the endolymph. The result of the analysis of the two parts of the jelly shows that it must be secreted with its own special composition, and is not merely an intermediate between serum and sea water. It may be noted that the secretion of the jelly probably occurs in the ampullae themselves, and not along the tubes, which are lined by a tough connective tissue layer (Dotterweich, 1932).

The excess of chloride over sodium + potassium is high (mean of all fish, 136 mM/kg water) but a similar argument holds here as for the endolymph. The artificial "jelly" solution is hypertonic to the jelly itself, and it is therefore probable that some of the cation deficit is made up by weak bases such as a protein or TMO rather than by the magnesium used in the artificial solution. But the conductivity suggests that there is a considerable amount of magnesium (+ calcium) in the jelly, and this is not unreasonable because although most elasmobranch body fluids contain little magnesium, perivisceral coelomic fluid contains as much as 20 mM magnesium (Hartman *et al.*, 1941) and there is 10 Ca and 54 Mg Mm/kg water in sea water.

The biological function of the jelly is not known for the function of the sense organ as a whole remains uncertain. It is known that the ampullae are sensitive to temperature changes (Sand, 1938), but the jelly does not have a specially high thermal conductivity (Murray, 1960a). It is known that the ampullae are also sensitive to small tactile stimuli applied at the opening of the tubes and therefore the jelly must act as a mechanical link in the chain of stimulation, but there is also the possibility that the ampullae are receptors for weak voltage gradients or electric pulses in the water, whether produced by the animal's own electric organs or not (Murray, 1960a, b). The electrical conductivity of the jelly may therefore be an important property, and its high value, nearly equal to that of sea water and twice as high as that of the body fluids, provides a possible justification for the anatomical arrangement of the sense organs. The system of tubes ending

widespread over the dorsal and ventral surfaces of the body would act like insulated conductors testing the sea-water potentials over as wide an area as possible.

## APPENDIX

TABLE A1—CONCENTRATION OF SODIUM IN THE BODY FLUIDS (mM/kg WATER)
(All fish *Raja clavata* except 14, *R. montagui* and 17, *R. brachyura*)

| Fish | Serum | Cranial fluid | Peri-lymph | Endolymph | | Lorenzini jelly | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Jelly | Fluid | First | Second | Mixed |
| 1 | | | 287 | 298 | | | | |
| 3 | | | 270 | 339 | | | | |
| 6 | | | 283 | 257 | | | | |
| 7 | | | 256 | 272 | | | | |
| 8 | | | 269 | 269 | | | | |
| 9 | 299 | 286 | 289 | 310 | 338 | | | 462 |
| 10 | 293 | 273 | 275 | 292 | 325 | 412 | 465 | |
| 11 | 270 | 262 | 256 | 282 | | 425 | 422 | |
| 12 | | 266 | 271 | 289 | 293 | 436 | 436 | |
| 13 | 290 | 293 | 292 | 305 | | | | 467 |
| 14 | 268 | 256 | 260 | 285 | | 434 | 415 | |
| 15 | 258 | 267 | 268 | 281 | 341 | 439 | 470 | |
| 16 | 292 | 288 | 301 | 288 | 302 | 420 | 426 | |
| 17 | | | | | | 398 | 394 | |
| 18 | | | | | | 404 | 394 | |

TABLE A2—CONCENTRATION OF POTASSIUM IN THE BODY FLUIDS (mM/kg WATER)

| Fish | Serum | Cranial fluid | Peri-lymph | Endolymph | | Lorenzini jelly | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Jelly | Fluid | First | Second | Mixed |
| 1 | | | 3·05 | 60·0 | | | | |
| 3 | | | 3·98 | 57·7 | | | | |
| 6 | | | 3·80 | 68·7 | | | | |
| 7 | | | 3·33 | 65·2 | | | | |
| 8 | | | 3·38 | 66·8 | | | | |
| 9 | 4·53 | 3·95 | 4·26 | 70·3 | 81·3 | | | 14·0 |
| 10 | 3·82 | 3·13 | 3·04 | 62·1 | 63·2 | 11·2 | 11·6 | |
| 11 | 3·92 | 3·15 | 3·86 | 64·2 | | 11·7 | 13·8 | |
| 12 | | 2·97 | 3·04 | 60·8 | 56·8 | 12·8 | 13·8 | |
| 13 | 4·28 | 3·38 | 3·36 | 61·0 | | | | 11·9 |
| 14 | 2·30 | 2·59 | 2·26 | 60·1 | | 11·4 | 12·2 | |
| 15 | 3·20 | 3·20 | 2·88 | 64·1 | 72·1 | 12·9 | 14·6 | |
| 16 | 3·37 | 3·08 | 3·15 | 59·4 | 66·0 | 11·7 | 12·9 | |
| 17 | | | | | | 13·7 | 17·0 | |
| 18 | | | | | | 12·7 | 13·7 | |

TABLE A3—CONCENTRATION OF CHLORIDE IN THE BODY FLUIDS (mM/kg WATER)

| Fish | Serum | Cranial fluid | Peri-lymph | Endolymph jelly | Lorenzini jelly | | |
|---|---|---|---|---|---|---|---|
| | | | | | First | Second | Mixed |
| 9 | 314 | 315 | 344 | 347 | | | 613 |
| 10 | 287 | 308 | 304 | 384 | 544 | 558 | |
| 11 | 296 | 276 | 290 | 399 | 585 | 575 | |
| 12 | | 320 | 333 | 413 | 612 | 630 | |
| 13 | 337 | 339 | 343 | 408 | | | 600 |
| 14 | 290 | 260 | 247 | 345 | 540 | 521 | |
| 15 | 305 | 286 | 298 | 366 | 471 | 439 | |
| 16 | 319 | 318 | 326 | 390 | 563 | 559 | |
| 17 | | | | | 570 | 575 | |
| 18 | | | | | 570 | 585 | |

TABLE A4—CONCENTRATION OF UREA IN THE BODY FLUIDS (mM/kg WATER)

| Fish | Serum | Cranial fluid | Peri-lymph | Endolymph jelly | Lorenzini jelly | | |
|---|---|---|---|---|---|---|---|
| | | | | | First | Second | Mixed |
| 9 | 419 | 407 | 422 | 374 | | | 77 |
| 10 | 429 | 410 | 448 | 370 | 42·3 | 65·6 | |
| 11 | 487 | 475 | 472 | 378 | 38 | 60·8 | |
| 12 | | | | | | | |
| 13 | 469 | 475 | 477 | 413 | | | 99·5 |
| 14 | 476 | 463 | 457 | 360 | 76 | 105 | |
| 15 | | | | | | | |
| 16 | 418 | 418 | 416 | 370 | 74 | 114 | |
| 17 | | | | | 77·3 | 124 | |
| 18 | | | | | 61·4 | 92 | |

*Acknowledgements*—We should like to thank the Director and Staff of the Marine Biological Association's Laboratory at Plymouth for their hospitality and for providing the fish and other facilities, and Dr. G. Howells of the Ministry of Agriculture, Fisheries and Food for her help in determining the freezing points.

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## SHORT COMMUNICATION

# THE RESTING POTENTIAL AND POTASSIUM LEVELS OF CELLS FROM ACTIVE AND INACTIVE SNAILS

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(*Received* 23 *August* 1960)

**Abstract**—Nerve cells from active snails have a higher resting potential and potassium concentration than cells from inactive snails.

ACTIVE garden snails (*Helix aspersa*), if kept in the laboratory for some time, often settle down to an inactive condition. They retract into their shells and the shell opening becomes covered by a thin epiphragm of dried mucus. During the course of a series of experiments on the physiology and pharmacology of the snail brain, we were struck by the differences in the properties of brains taken from active as compared with inactive snails. In particular the inactive snails seemed to have a lower resting potential in their nerve cells. This was checked by the following experiment.

A series of brains was dissected out from inactive snails and the connective tissues around the brain partially removed. The brains were placed in 70% Ringer solution (Cardot, 1921), observed individual nerve cells in the visceral and parietal ganglia were penetrated with microelectrodes, and their resting potentials measured. The resting potentials had the frequency distribution shown in Fig. 1 (a), the majority having a resting potential of 10–15 mV. A similar series of experiments was carried out on brains taken from active snails. These nerve cells had a different frequency distribution of resting potential to those from inactive snails, Fig. 1 (b), with a mean potential between 15 and 25 mV.

Though there was an overlap between the two distributions, the resting potentials of the cells from the inactive snails were in general lower than those from the active snails. Snails brought out of their inactive state by immersing in water showed a similar resting potential distribution to that of inactive snails, as did also nerve cells from snails resting after feeding. On the other hand snails that had become active due to exposure to artificial rain showed resting potentials of the type (b) distribution curve.

The resting potentials recorded in these snails were on the whole much lower than those described by Tauc (1955) from the brain of the snail *Helix pomatia* where he found that the resting potential varied from 30 to 50 mV.

Another difference that was noted between the brains of active and inactive snails was that the active snails showed a higher number of cells that were

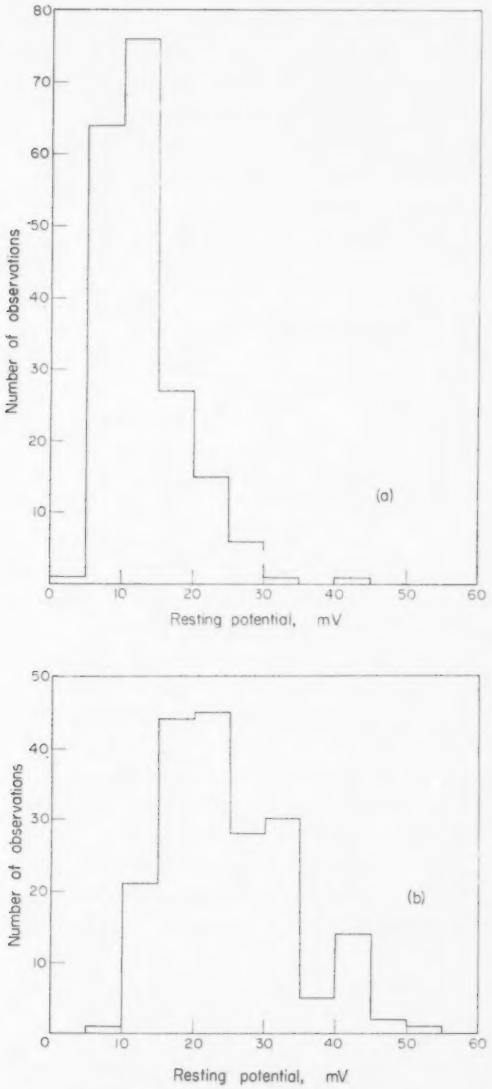


FIG. 1. Frequency distribution of resting potentials from brain cells of *Helix aspersa*. (a) Potentials from inactive snails. (b) Potentials from active snails.

spontaneously active. The most active preparations would show 30 per cent of the cells with spontaneous action potentials (though not all of these showed an

overshoot of the base line). In the brains from inactive snails there was very little spontaneous activity, only approximately 1 per cent of the speared cells showed spontaneous activity. This may also be linked to the fact that the resting potential in the active snails' nerves was somewhat higher.

In all the preceding experiments, the isolated brains and nerve cells were bathed in physiological saline of the same concentration. If the resting potential in the snail nerves is mainly due to the difference between the concentration of the potassium ions inside and outside the nerve cells (Hodgkin, 1951; Tauc, 1958) then the observed difference between the resting potentials could be due to a difference in the potassium concentration inside the nerve cells of the two groups of animals. To study this a series of brains was isolated from resting snails, trimmed, washed and homogenized in distilled water. The potassium concentration was then determined by means of a flame photometer. A similar series of experiments was carried out on brains isolated from inactive snails. The results are shown below (ten series of separate experiments being carried out in each case).

Potassium concentrations in *Helix* brain

| | |
|---|---|
| Brains from inactive snails | $2{\cdot}87 \pm 0{\cdot}20$ m equiv K/100 g brain |
| Brains from active snails | $3{\cdot}64 \pm 0{\cdot}23$ m equiv K/100 g brain |

The active snails' brains have a significantly higher potassium concentration than do brains from inactive snails ($0{\cdot}05 < P < 0{\cdot}02$). This agrees with the fact that the brains of the active animals have a higher resting potential. The observed potassium concentrations are very much lower than those obtained by Steinbach & Spiegelman (1943) for squid axoplasm, but in the present case, the isolated brains contained a high proportion of connective tissue and extracellular fluid, both of which would tend to decrease the observed values for potassium.

Previous investigators, Hoyle (1954), Hughes & Kerkut (1956) and Kerkut & Taylor (1956) have described the way in which variations in the concentration of solutions bathing a tissue can affect its reactions and behaviour. The present experiments suggest that variations can also occur in the intracellular components and that these may be correlated with the behaviour of nerve cells and possibly the behaviour of the whole animal.

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# BOOK REVIEWS

**Data for Biochemical Research.** Edited by R. M. C. DAWSON, DAPHNE ELLIOTT, W. H. ELLIOTT and K. M. JONES. Clarendon Press, Oxford, 1959. xii + 299 pp., 63s.

THE Chapter headings are (1) Table of Biochemical Compounds (Amino acids, Peptides; Carboxylic acids; Purines; Carbohydrates; Lipids; Steroids; Vitamins; Porphyrins; Chlorophylls; Caroteinoids; Enzyme inhibitors; Metal chelating compounds; Antimetabolites; Drugs; Macromolecules). (2) Enzymes commonly used as laboratory reagents. (3) Buffers and physiological media. (4) Methods for detection of biochemical compounds on paper. (5) The properties of some ion exchange resins. (6) Isotopic data. (7) Manometry. (8) Nomograms for manometer constants. (9) Ammonium sulphate solutions. (10) Miscellaneous reagents. (11) Acid base indicators. (12) Table of normalities. (13) Autoclave temperature-pressure conversion table. (14) Miscellaneous formulae. (15) International Atomic Weights.

It is of course impossible in a mere 299 pages to please everyone, but the Editors (and the twelve other contributors) have done a very good job in presenting essential data. Not only do they give this but they also give full references where to find further information. Thus the tables of biochemical compounds have headings; Name, Synonyms, Formula; Mol. Wt.; Physical form; Melting Point; Preparation references; Estimation references; Derivatives. General Remarks; Reviews etc. Personally I found that one of the most useful sections was that on the methods for detecting biochemical compounds on paper. This is a very useful guide to the chemical tests for visualizing spots after chromatography. Full details of each chemical test are given together with critical references to the literature. In fact Chapter 4 could well be published as a paper-back separate and would no doubt be in great demand as a laboratory guide. One weak section is that dealing with Physiological media. The authors give only the most obvious recipes, i.e. the various Krebs-Ringers, Frog Ringer, Locke and Tyrodes. There is no mention of any of the other suitable salines. On the other hand details are given for the preparation of twenty-seven different buffer systems.

To sum up, this volume will be an essential reference book, both for the laboratory and the library.

G. A. KERKUT

D. and K. STANLEY-JONES: **The Kybernetics of Natural Systems: A Study in patterns of Control.** Pergamon Press, London, 1960. x + 145 pp., 42s.

THE title of this book will, no doubt, attract many people, who want to know more about the difficult subject of the application of cybernetics to biological systems. They will be disappointed if they hope for a mathematical approach, for this book in no way even tries to evaluate any quantitative aspects of feed-back in biological systems. Instead the book enumerates a number of systems, both living and non-living in which feed-back might be present. Among these are several which obviously do not belong to this class, if one applies to them the writers' own definition, namely that feed-back comprises a portion of the output which is algebraically added to the input. This definition surely excludes the pulsating stars, for if it is rightly claimed that the flare-up is caused by a nuclear reaction and the restoration in size by the gravitational field, what is being fed back ? It is rightly pointed out that when negative feed-back fails all kinds of ills may beset the system, which may be identifiable with diseases. However, the writers go much too far in this respect, by implying that any failure in such a system must be due to feed-back failure. Thus, all tremors are ascribed to this cause, overlooking cases such as changes in calcium metabolism caused by hormone imbalance, in which it is presumably not the feed-back system, but the main system which is affected. In general, there is an unfortunate tendency to make statements of "fact", which vary from truisms to outright falsehoods, thus burying those instances for which a good case could be made. Hence the value of this book is in this respect considerably diminished. Nevertheless, for people interested in the subject, this may be found to be a challenging book, with its metaphysiological approach which embraces "neural dynamics", "kybernetic anatomy" and a host of other, often ill-defined terms. To decide in which cases one can agree with the authors' viewpoint, and in which they must definitely be discarded for some concrete reason, may serve to clarify the problem in one's own mind. At the same time, one may determine whether these views extend the insight given by Cannon's homeostasis principle and his "wisdom of the body". Only if this is done can one evaluate Norbert Wiener's prediction that this work "is destined to occupy a classical place". In many respects this book may make enjoyable reading for those whose interest in this field is unspecialized, and who therefore will not be dismayed by the lack of definitions and the lapses in rigorous attention to accuracy. The style is light and pleasant and the book is divided into well-organized sections. It perhaps provides a necessary introduction to the more serious, and largely unexplored, field of quantitative study in cybernetics as applied to biology.

C. A. G. WIERSMA

E. LESTER SMITH: **Vitamin $B_{12}$.** Methuen, London; John Wiley, New York, 1960. 196 pp., 15s.

THIS is the only monograph to cover the whole field of vitamin $B_{12}$ research. The author traces the independent investigations of seemingly unrelated factors which finally led to the isolation and crystallization of vitamin $B_{12}$ in 1948 by Folker's and the author's groups. Every aspect of vitamin $B_{12}$, its distribution, assay, isolation, chemistry, metabolism, and clinical application are concisely treated with adequate references after each chapter. Although the major advances by Dr. Barker's group could not be included, the critical evaluation of vitamin $B_{12}$ research to 1959 makes this an invaluable survey. The problems raised in the discussion on biochemical function are still relevant.

J. DUBNOFF

*Comp. Biochem. Physiol.*, 1961, *Vol.* 2, *pp.* 81 *to* 89. *Pergamon Press Ltd., London. Printed in Great Britain*

# ATP-ase ACTIVITIES OF ADDUCTOR AND BYSSUS RETRACTOR MUSCLES OF MARINE MOLLUSCS, *MYTILUS* AND *MODIOLUS*

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(*Received* 23 *May* 1960)

**Abstract**—Proteins analogous to myosin B of rabbit striated muscle were extracted from adductor and byssus retractor muscles of marine molluscs, *Mytilus edulis* and *Modiolus demissus*. These proteins were purified with ordinary precipitation–dissolution process (repeated three times) and ultracentrifugation. The ATP-ase activity of all these proteins was enhanced markedly by addition of $CaCl_2$. On the other hand, the ATP-ase of adductor muscle protein was inhibited by $MgCl_2$, while that of byssus retractor muscle was activated more or less by $MgCl_2$. These proteins dephosphorylated not only ATP, but also ITP and ADP. Activation of ATP-ase on addition of a sub-equivalent concentration of PCMB was not marked in contrast to muscle myosin B. A high concentration of PCMB inhibited the ATP-ase. Intensity of light scattering was decreased markedly on addition of ATP in the case of *Modiolus* adductor and byssus retractor proteins, while the decrease was not observed in *Mytilus* proteins.

## INTRODUCTION

THE adductor and byssus retractor of marine molluscs, i.e. *Mytilus* and *Modiolus*, are classified as smooth muscles. Contractile tension of these smooth muscles is of the same magnitude as that of vertebrate striated muscles, but it is maintained for much longer periods than that of vertebrate striated muscles. The large amount of paramyosin in these smooth muscles may be related to a "catch mechanism".

In this report, however, experiments are confined to the proteins which were extracted with Weber–Edsall solution (pH ca. 8·0) and purified with ordinary repeated dissolution–precipitation processes and ultracentrifugation. Some comparative studies of ATP-ase and of the physical properties of these proteins analogous to rabbit muscle myosin B will be reported here.

## MATERIALS AND METHODS

The posterior adductor muscle and anterior- and posterior-byssus retractor muscle of *Mytilus edulis* and *Modiolus demissus* were used throughout the experiments.

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These muscles were excised and rinsed at least twice with artificial sea water of the following composition:

| | |
|---|---|
| NaCl | 423·0 m-equiv./l. |
| KCl | 9·0 m-equiv./l. |
| $CaCl_2$ 2 $H_2O$ | 9·27 m-equiv./l. |
| $MgCl_2$ 6 $H_2O$ | 22·94 m-equiv./l. |
| $MgSO_4$ 7 $H_2O$ | 25·50 m-equiv./l. |
| $NaHCO_3$ | 2·15 m-equiv./l. |

The pH of the solution is about 7·8. The muscles were ground by Waring blender, and were then extracted overnight with cold (4°C) Weber–Edsall solution, i.e. KCl, 0·6 M; $Na_2CO_3$, 0·01 M; $NaHCO_3$, 0·04 M (pH around 8·0). The extracted solution was then centrifuged at 8000 *g* for 15 min and the supernatant was diluted with redistilled water about seven-fold. Rapid precipitation was observed as in rabbit myosin B. Since the ionic strength of this diluted solution is about 0·1 and the pH is greater than 7, a large amount of paramyosin may remain in the supernatant (Johnson *et al.*, 1959). The precipitate was collected by centrifugation at 3000 *g* for 10 min and dissolved in concentrated KCl (final concentration, 0·6 M KCl). After a centrifugation at 3000 *g* for 10 min, the supernatant was again diluted in redistilled water, etc. By repeating such precipitation–dissolution three times at pH about 7·0 and after final ultracentrifugation at 3500 *g* for 1 hr, almost all proteins which are insoluble in 0·6 M KCl and soluble in water at pH around 7·0 can be excluded. Paramyosin, therefore, is probably not a major component of our purified proteins. The concentration of the final preparation was generally about 2 mg/ml, and the solution was diluted for ATP-ase or light scattering measurements. The protein concentration was determined by the method of Lowry *et al.* (1951).

The proteins thus purified showed fairly strong flow birefringence when examined between two cross-polarized polaroid plates.

The amount of inorganic phosphate which was liberated by these proteins from ATP was measured chiefly by the Fiske–Subbarow method (1925); in some experiments the ATP-ase activity was measured with a pH-stat (Titrator, Radiometer, Copenhagen, Type TTT lb).

The angular distribution of light-scattering intensities at different angles was measured with a Brice-Phoenix Universal Light Scattering Photometer (Model 1000). The refractive index increment which is needed in the calculation of molecular weight was measured with a Brice-Phoenix Differential Refractometer.

Original stock protein solutions were diluted in 0·6 M KCl, 0·1 M tris buffer (pH 7·0 and 8·0) for ATP-ase or light-scattering experiments.

## RESULTS

### *Adductor muscle protein*

Addition of $CaCl_2$ brings about activation of ATP-ase of adductor muscle protein either from *Mytilus* or *Modiolus*. The greatest activation was observed

on addition of $CaCl_2$ of around $10^{-2}$ molar (Fiske–Subbarow method). When the ATP-ase activity was measured by pH-stat, the inorganic phosphate liberation was generally greater in $10^{-1}$ M than in $10^{-2}$ M $CaCl_2$. The reason for this

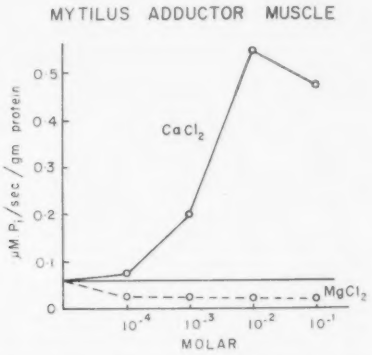
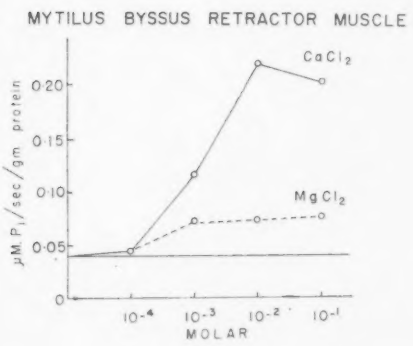
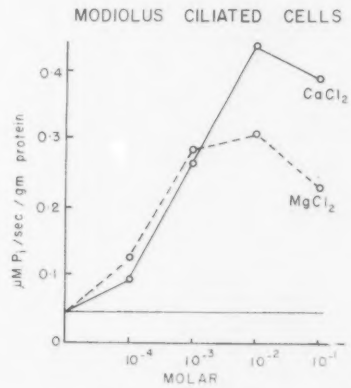



FIG. 1. The effect of divalent ions on the rate of ATP-ase activity of adductor muscle protein from *Mytilus*. $P_i$, inorganic phosphate released. The protein (0·062 per cent in final concentration) was dissolved in 0·6 M KCl + 0·1 M tris buffer (pH 8·02). ATP ($10^{-3}$ M in final concentration) in 0·6 M KCl + 0·1 M tris, pH 8·02. The rate of dephosphorylation was determined from the average of inorganic phosphate released at three or four different time points. Temperature, 25°C.




(2a)




(2b)

FIG. 2 (a). The effect of divalent ions on the rate of ATP-ase activity of byssus retractor from *Mytilus*. The protein was dissolved in 0·6 M KCl + 0·1 M tris buffer (pH 8·02). Final concentration of the protein was 0·0158 per cent. ATP, $10^{-3}$ M in final. Temperature, 25°C.

FIG. 2 (b). The effect of divalent ions on the rate of ATP-ase activity of protein from isolated cells of *Modiolus* gill. The protein was not purified enough, i.e. only one precipitation–dissolution and centrifuged at 8000 *g* for 15 min. ATP, $10^{-3}$ M in final concentration. Protein concentration was 0·032 per cent. Temperature, 25°C.

difference in result when using these two methods remains to be explained. On the other hand, $MgCl_2$ inhibited the activity to less than a half of that obtained without $MgCl_2$ (Fig. 1).

*Byssus retractor muscle protein*

Addition of $CaCl_2$ resulted in activation of ATP-ase of byssus retractor muscle protein either from *Mytilus* or from *Modiolus*. In this case, too, the activation was

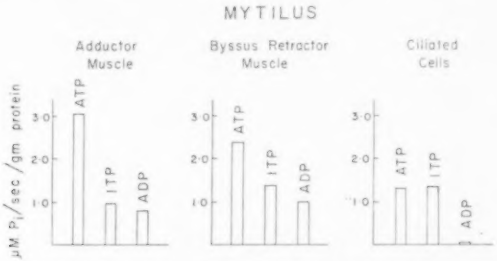



FIG. 3. The rates of dephosphorylation of ATP, ITP and ADP are compared among three proteins, i.e. adductor, byssus retractor, and ciliated cells from gills of *Mytilus*. ATP concentrations were $10^{-3}$ M, $10^{-3}$ M and $5 \times 10^{-4}$ M respectively. Protein concentrations were 0·0138, 0·0125 and 0·0248 per cent respectively. $CaCl_2$, $10^{-2}$ M for each. Temperature, 25°C.

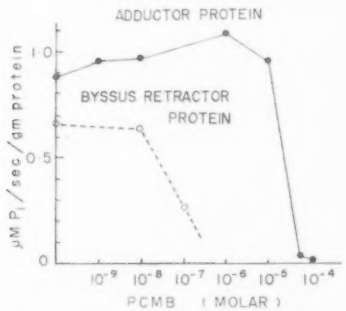



FIG. 4. The rates of dephosphorylation of ATP at different concentration of PCMB. Adductor protein from *Mytilus* (concentration, 0·0138 per cent) was dissolved in 0·6 M KCl+0·1 M tris (pH 8·0). Byssus retractor protein from *Mytilus* (concentration, 0·0126 per cent) was dissolved in 0·6 M KCl+0·1 M tris (pH 8·0). ATP, $10^{-3}$ M in final concentration. $10^{-2}$ M $CaCl_2$ was added for each as an activator. Temperature, 25°C.

greatest in $10^{-2}$ M $CaCl_2$ when using the Fiske–Subbarow method, but was still greater in $10^{-1}$ M when using the pH-stat method. $MgCl_2$, in contrast to adductor muscle protein, activated to some extent the ATP-ase of retractor muscle protein (Fig. 2).

*Inosinetriphosphatase and adenosinediphosphatase activity*. Proteins extracted from adductor and byssus retractor muscle of *Mytilus* dephosphorylated not only ATP, but also ITP and, to some extent, ADP (Fig. 3).

On the other hand, the protein extracted from ciliated gill apparatus (ciliated cells were isolated from gill by 0·1 per cent trypsin; later 0·1 per cent ovomucoid was added to stop the trypsin reaction; the protein was purified once, not ultra-centrifuged) could not dephosphorylate ADP, but it dephosphorylated ITP as fast as ATP.

*Effect of parachloromercuribenzoate* (*PCMB*). The pre-incubation of myosin B from rabbit muscle in dilute PCMB solution for a few minutes brings about a fairly strong activation (more than three times control activity) of its ATP-ase (Kielley & Bradley, 1956). Such an activation by PCMB-pre-incubation, however, was not at all marked in the case of the proteins from adductor and byssus retractor muscles of *Mytilus* (Fig. 4).

*Light-scattering characteristics.* The intensities of scattered light at different angles, $\theta$, from adductor muscle and byssus retractor muscle protein solutions are compared in Fig. 5 (cf. Zimm, 1948). $K$ in these figures is a constant for each protein, which can be expressed as follows:

$$K = \frac{32\pi^3}{3\lambda^4}\frac{n_0^2}{N}\frac{(n-n_0)^2}{c^2}$$

where

$n_0$ = refractive index of the solvent in which the proteins were dissolved (0·6 M KCl + 0·1 M tris buffer, pH 8·0).

$\lambda$ = wavelength of the light used, i.e. 436 m$\mu$.

$N$ = Avogadro number.

$c$ = concentration of the protein in g/ml.

$\frac{n-n_0}{c}$ = refractive index increment which was measured for each protein.

$R$ = intensity of scattered light at different angles, which was calculated after several ordinary geometrical corrections related to the apparatus.

The intercept of the curve at zero angle is equal to the reciprocal to the weight average molecular weight, $\bar{M}_w$, while the intercept extrapolated from large angles is equal to the reciprocal to the number average molecular weight, $\bar{M}_n$ (Benoit, 1953).

On addition of $10^{-3}$ M ATP (final concentration) a marked decrease in intensity of scattered light was observed in *Modiolus* myosin, i.e. adductor and byssus retractor protein. It is worth while to note that the weight average molecular weight of these two proteins seems to be unchanged by ATP-addition, although number average molecular weight was decreased. Such response is very similar to that of rabbit myosin (Gellert *et al.*, 1959).

The size of molecules can be estimated roughly as the average radius of gyration, $\bar{r}_g$, defined in the Zimm plot as

$$\bar{r}_g = \frac{\sqrt{3}\lambda}{4\pi n}\sqrt{\frac{\text{intercept}}{\text{slope}}}$$

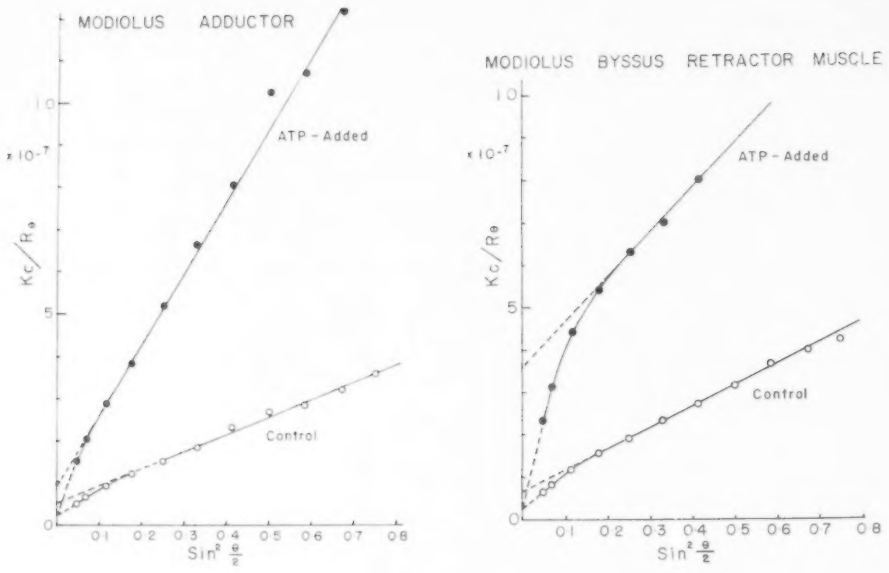



FIG. 5. Zimm plot of 24-hr extracted *Modiolus* adductor (a) and byssus retractor protein (b) before (lower curves) and after (upper curves) ATP-addition. Protein concentrations were 0·022 and 0·014 per cent respectively in 0·6 M KCl+0·1 M tris (pH 7·4). Extrapolation from low angles gives weight average molecular weight and the one from larger angles gives number average molecular weight. Both proteins from *Modiolus* show large decrease in light-scattering intensity on application of $10^{-3}$ M ATP. Temperature, 23°C.

TABLE 1—EFFECT OF $10^{-3}$ M ATP ON THE MOLECULAR WEIGHT AND RADIUS OF GYRATION OF *Modiolus* AND *Mytilus* PROTEINS

| Source of protein | $\bar{M}_w$ Weight average molecular weight | $\bar{M}_n$ Number average molecular weight | $\bar{r}_g$ Average radius of gyration, Å |
|---|---|---|---|
| *Modiolus* adductor muscle | | | |
| before ATP-addition | $5{\cdot}0 \times 10^7$ | $1{\cdot}89 \times 10^7$ | 1250 |
| after ATP-addition | $5{\cdot}0 \times 10^7$ | $1{\cdot}18 \times 10^7$ | 2025 |
| *Modiolus* byssus retractor muscle | | | |
| before ATP-addition | $4{\cdot}0 \times 10^7$ | $2{\cdot}83 \times 10^7$ | 1210 |
| after ATP-addition | $4{\cdot}0 \times 10^7$ | $1{\cdot}49 \times 10^7$ | 1370 |
| *Mytilus* adductor muscle | | | |
| before and after ATP-addition | $3{\cdot}10 \times 10^6$ | $3{\cdot}10 \times 10^6$ | 3900 |
| *Mytilus* byssus retractor muscle | | | |
| before and after ATP-addition | $2{\cdot}90 \times 10^6$ | $9{\cdot}80 \times 10^5$ | 2440 |
| *Mytilus* ciliated cells of gill | | | |
| before and after ATP-addition | $1{\cdot}74 \times 10^7$ | $1{\cdot}74 \times 10^7$ | 5090 |

The average radius of gyration is increased to some extent by ATP-addition as is that of rabbit myosin B. It may be noteworthy that the amount of decrement of light-scattering intensity due to ATP-addition was almost the same whether $CaCl_2$ was added or not, while ATP-ase activity was markedly enhanced by $CaCl_2$.

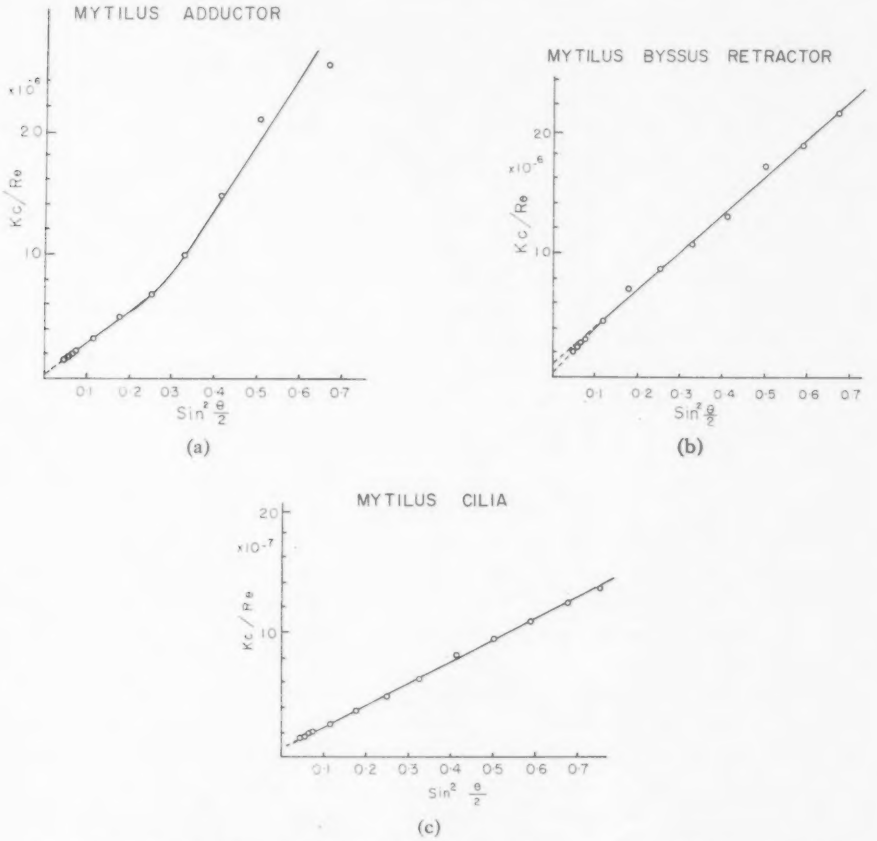



FIG. 6. Zimm plot of 24-hr extracted *Mytilus* adductor, byssus retractor and ciliated gill cells. Protein concentrations were 0·025, 0·0138 and 0·0226 per cent respectively in 0·6 M KCl+0·1 M tris (pH 8·0). In contrast to the proteins of *Modiolus* the light-scattering intensity did not change on ATP-addition. Temperature, 23°C.

In other words, $CaCl_2$ did not enhance the decrease of light-scattering intensity brought about by ATP. These results are summarized in Table 1.

On the other hand, proteins from *Mytilus* did not show any decrease in light-scattering intensity on addition of ATP (Fig. 6). The weight average molecular weights of these proteins are smaller, to some extent, than those of *Modiolus*. Whether such a qualitative difference in light-scattering characteristics between

these two marine molluscs is due to species difference or to seasonal variation (*Modiolus*, November and December; *Mytilus*, January to March) or to differences in extractability remains to be proved.

## DISCUSSION

Proteins which were extracted from adductor muscle, byssus retractor muscle and ciliated gill cells of *Mytilus* and *Modiolus* all dephosphorylated ATP at a rate which was accelerated markedly by $CaCl_2$. On the other hand, $MgCl_2$ activated the ATP-ase of retractor muscle (this confirms Kahn & Johnson's result, 1960) and of ciliated gill cells, and inhibited the ATP-ase activity of adductor muscle. These proteins dephosphorylated not only ATP, but also ITP and, to some extent, ADP.

The ATP-ase of adductor and byssus retractor of *Mytilus* exhibited only small or practically no sulfhydryl reagent activation in contrast to rabbit myosin B. They did not dephosphorylate sodium pyrophosphate. According to Nelson (1959) the ATP-ase of *Mytilus* sperm tail exhibits no sulfhydryl reagent activation, but dephosphorylates sodium pyrophosphate.

Although ATP-ase activity seems to be similar in *Mytilus* and *Modiolus*, the light-scattering properties are quite different, i.e. addition of ATP decreased markedly the light-scattering intensity of *Modiolus* adductor and byssus retractor proteins, while it did not in *Mytilus* proteins. Addition of rabbit actin, i.e. 2 mg rabbit actin to 0·84 mg *Mytilus* adductor myosin and 1 mg rabbit actin to 0·41 mg *Mytilus* byssus retractor myosin, had no detectable influence on the light-scattering intensity. According to Kahn & Johnson (1960), byssus retractor muscle of *Mytilus* did not show any viscosity drop on addition of $4 \times 10^{-3}$ M ATP. They suggested, therefore, that there is no detectable amount of actin in this protein. The weight average molecular weights of these two proteins of *Mytilus* were less than those of *Modiolus*.

Proteins extracted from *Mytilus* and *Modiolus* are similar to rabbit myosin B in some aspects, but quite different in others. Even in *Mytilus* or *Modiolus* itself the ATP-ase activities are different among adductor, byssus retractor, ciliated gill cells, and sperm tail. The correlation between the difference in enzymatic and physical properties among these proteins and their physiological functions remains to be studied.

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# MÉTABOLISME DE $^{131}$I ET FONCTION THYROÏDIENNE CHEZ LA LARVE (AMMOCOETE) D'UN CYCLOSTOME, *PETROMYZON PLANERI* BL.

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**Résumé**—(1) Les larves de *Petromyzon planeri* Bl. (Ammocoetes) fixent les iodures marqués par $^{131}$I ajoutés à l'eau dans laquelle ils sont immergés. Ce processus, assez intense pendant 4 jours a +18°C, devient par la suite beaucoup moins actif. $^{131}$I est concentré préférentiellement dans la région de l'endostyle et dans la chorde dorsale. L'endostyle renferme une ébauche thyroïdienne fonctionnelle dont l'activité a été étudiée par radiochromatographie et radioélectrophorèse des constituants iodés marqués de la région endostylaire et des tissus récepteurs des hormones.

(2) La région endostylaire renferme de la thyroxine, de la 3 : 5 : 3′-triiodothyronine et leurs précurseurs, la 3 : 5-diiodotyrosine et la 3-monoiodotyrosine, incorporées dans une protéine, d'où les libère une hydrolyse protéinasique. On ne trouve, par contre, aucune iodoprotéine dans les tissus récepteurs (ensemble des tissus mous en dehors de l'endostyle), dont la thyroxine et de petites quantités de 3 : 5 : 3′-triiodothyronine peuvent être extraites sans hydrolyse protéinasique.

(3) Les rapports quantitatifs entre les acides aminés marqués de la région endostylaire et des tissus récepteurs sont très variables suivant le temps d'incubation avec $^{131}$INa. L'hormonogénèse, bien que plus lente, relève d'un même mécanisme dans l'endostyle des larves du Cyclostome étudié et dans le corps thyroïde des Mammifères.

(4) Le transport sanguin des hormones paraît être assuré par une protéine plasmatique capable de fixer la thyroxine.

**Abstract**—(1) $^{131}$I labelled iodides, added to immersion water, are fixed by larvae of *Petromyzon planeri* Bl. (Ammocoetes). The kinetics of binding and the repartition of radioactivity in tissues of Ammocoetes were studied. $^{131}$I is concentrated preferentially in two organs, the endostyle and the dorsal-cord. Endocrine activity of the endostyle was studied by radio-chromatography and radio-electrophoresis of labelled iodinated compounds from the endostylar region as well as tissues receiving hormones.

(2) The endostylar region contains thyroid hormones, thyroxine and 3 : 5 : 3′-triiodothyronine, together with their precursors, 3 : 5-diiodotyrosine and 3-monoiodotyrosine. These iodinated aminoacids are incorporated into a protein from which they are liberated by enzymatic hydrolysis.

No iodoprotein is present in non-endostylar tissues; almost all of the radioactivity is extractable by acid *n*-butanol. Receptive tissues contain only thyroxine and small amounts of 3 : 5 : 3′-triiodothyronine.

(3) The quantitative ratio of iodinated aminoacids, both in the endostyle and in peripheric tissues, is highly variable according to incubation time with labelled iodides. The biosynthesis of thyroid hormones by the endostyle of Ammocoetes seems to be not dissimilar to that of the thyroid gland of higher Vertebrates but it is, however, rather slower.

(4) The haematic transport of the thyroid hormones is assured by a thyroxine binding protein.

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PARMI les Vertébrés inférieurs, les Cyclostomes renferment, à l'état larvaire, un organe glandulaire sous-pharyngien, l'endostyle, dont une partie donne naissance, lors de la métamorphose, à un corps thyroïde bien différencié (Marine, 1913; Kraentzel, 1933; Leach, 1939). La fonction endocrine de l'endostyle a été d'abord envisagée à partir de données embryologiques (Dohrn, 1875, 1885), puis niée (Rémy, 1922; Horton, 1934; Stokes, 1939). Un ensemble de recherches plus récentes ont montré la fixation d'iodures marqués par certaines régions de cet organe (Gorbman & Creaser, 1942; Barrington & Franchi, 1956) et la formation dans celui-ci d'acides aminés iodés (Leloup & Berg, 1954; Leloup, 1955). Aucun travail biochimique d'ensemble n'ayant été entrepris à ce sujet, nous avons poursuivi des recherches sur divers aspects du métabolisme de l'iode ches les larves de *Petromyzon planeri* Bl. ayant séjourné dans l'eau additionnée d'iodures marqués par $^{131}$I.

Après avoir étudié la cinétique de la fixation des iodures marqués par les larves et la répartition d'$^{131}$I dans leurs différents tissus, nous nous sommes proposé d'identifier les constituants iodés présents dans la région endostylaire et dans les tissus récepteurs. Comme la sécrétion endostylaire est holocrine (Gorbman, 1955), une protéine capable de fixer la thyroxine pourrait jouer un role dans la résorption des hormones au niveau du tube digestif: nous avons donc recherché si le sérum des larves renferme des protéines plasmatiques analogues à la TBP des Mammifères, susceptibles d'assurer le transport sanguin des hormones thyroïdiennes.

## PARTIE EXPÉRIMENTALE

### I. *Marche des Expériences et Techniques*

#### 1. *Fixation et répartition d'*$^{131}$I

200 ammocoetes, récoltés dans la rivière Sarno (v. Zanandrea, 1959), ont été adaptés à +18–20°C par un séjour de quelques jours en eau courante. Les larves ont été réparties par lots de 20 (environ 100 g) et placées à +18°C, sous aération continue, dans des récipients contenant 1500 ml d'eau additionnée de 300 $\mu$C $^{131}$INa sans entraîneur (activité spécifique: 5 mC/$\mu$g).

Des mesures de radioactivité du milieu d'immersion ont été faites toutes les 12 heures, pendant 8 jours, à l'aide d'un compteur à scintillation à cristal creux NaI (Tl), en vue d'établir la cinétique de la fixation du radioiode. $^{131}$I fixé a été calculé en tenant compte de la décroissance, en pour-cent de la quantité totale présente au temps zéro. Des contrôles directs de la fixation du radioiode ont été aussi opérés sur des larves sacrifiées en vue de cette dernière opération.

Après 4 ou 8 jours d'expérience, les animaux ont eté tués par décapitation au niveau de la 7ème branchie et saignés; la région endostylaire et la chorde dorsale ont été grossièrement disséquées. Nous avons ainsi obtenu 4 fractions, dont une partie aliquote a fait l'objet de mesures de radioactivité au compteur à scintillation: le sérum, la région endostylaire, la chorde dorsale et les autres tissus.

#### 2. *Analyse quantitative et qualitative des composés iodés marqués*

(a) *Techniques générales.* La séparation des iodures a été réalisée par électrophorèse sur papier Whatman 3 MM en solution tampon au carbonate d'ammonium-ammoniaque

(pH = 8,9, 4 V/cm, 2 h). L'analyse chromatographique mono- ou bidimensionnelle a été opérée sur papier Whatman n° 1 à +17°C, principalement avec les solvants suivants: (1) pentanol tertiairé saturé d'$NH_4OH$ 2N, sens ascendant, $t = 17°C$, 40 h; (2) *n*-butanol–acide acétique–eau (78 : 5 : 17), sens descendant, $t = 17°C$, 48 h. Les taches radioactives ont été localisées par autoradiographie (films Kodirex sans écran) et identifiées à l'aide de corps purs de référence incorporés dans le milieu ou de témoins marginaux des mêmes corps. Les corps de référence ont été révélés par la réaction de Pauly. La répartition quantitative d'$^{131}I$ entre les diverses fractions séparées par chromatographie a été déterminée à partir de mesures de la radioactivité des diverses taches. Celles-ci ont été réalisées soit au moyen deux compteurs de Geiger-Müller étroitement rapprochés se faisant face, entre lesquels les chromatogrammes ont été interposés, soit par un compteur à scintillation à cristal plat muni d'un écran de plomb à fenêtre de 1 cm × 1 cm.

(b) *Préparation des extraits en vue de l'analyse.* La région endostylaire de 20 ammocoetes a été homogénisée au broyeur de Potter-Elvehjem avec 10 ml $H_2O$ alcalinisée à pH = 8,5 à +4°C, et hydrolysée par les protéinases pancréatiques totales (pH = 8,5, 36 h, +38°C), puis par la papaïne activée au moyen d'$H_2S$ (pH = 5,0, 24 h, +38°C). Après centrifugation d'un résidu insoluble (1% d'$^{131}I$ environ), on lyophilise et délipide la poudre obtenue (40 ml d'éther déperoxydé additionnés de 2 ml d'éthanol absolu et d'une goutte de $NH_4OH$ conc.), que l'on met ensuite en suspension dans 5 ml HCl 0,1 N. On extrait à plusieurs reprises au *n*-butanol saturé d'HCl 0,1 N (25 ml). On élimine la phase aqueuse, aprés dosage des iodures marqués, et évapore à sec la phase butanolique ($t < +40°C$, atmosphère d'$N_2$). On reprend par le méthanol additionné d'$NH_4OH$ à 32 p. 100 (3 : 1), on sépare les iodures par électrophorèse et élue la bande demeurant à l'origine au cours de celle-ci, bande renfermant les combinaisons organiques iodées. Après purification par chromatographies préparatives et élutions successives, on opère l'analyse chromatographique mono- ou bidimensionnelle.

Les autres tissus sont traités de la même manière sans hydrolyse protéinasique préalable, la totalité d'$^{131}I$ qu'ils renferment ayant pu être extraite par le *n*-butanol chlorhydrique.

### 3. *Transport sanguin de la thyroxine*

Le sérum recueilli sur 20 animaux (1,5–2 ml) a été soumis à l'électrophorèse sur papier Whatman 3 MM, en présence de solution tampon véronal-véronal sodique de pH = 8,6, $\Gamma/2 = 0{,}075$ (5,2 V/cm de long, 0,4 mA/cm de large, 18 h) dans une série d'essais avec ou sans addition de L-thyroxine ($T_4$) marquée (0,1 $\mu$g $T_4$ par ml de sérum). Les électrophorégrammes des échantillons étudiés simultanément ont été, les uns colorés à l'Amidoschwartz 10 B (Bayer) pour localiser les protéines, les autres soumis à des mesures de radioactivité au chromatolecteur en vue d'une radioautographie ultérieure. La radioactivité a, en outre, été déterminée sur d'autres à l'aide d'un compteur à scintillation à cristal creux NaI (Tl) après découpage en bandes de 0,5 cm de large. En vue de s'assurer du transport de l'hormone endogène des essais analogues ont été poursuivis sur le sérum de larves ayant séjourné pendant 8 jours dans l'eau additionnée d'$^{131}INa$.

## II. *Résultats Expérimentaux*

### 1. *Cinétique de la fixation d'$^{131}I$ et répartition de la radioactivité entre les différents tissus*

On trouvera dans la Fig. 1 la courbe de fixation d'$^{131}I$ pendant 8 jours d'expérience, par les larves de *Petromyzon planeri* Bl. La fixation d'$^{131}I$, plus intense dans les 4 premiers jours, porte, dans l'essai décrit ci-dessus, sur 22,6% des iodures en 8 jours. Le taux d'$^{131}I$ dans les organismes est alors environ 3 fois plus élevé que dans le milieu extérieur.

La répartition de l'$^{131}$I aux deux temps successifs de l'expérience a été reportée dans le Tableau 1.

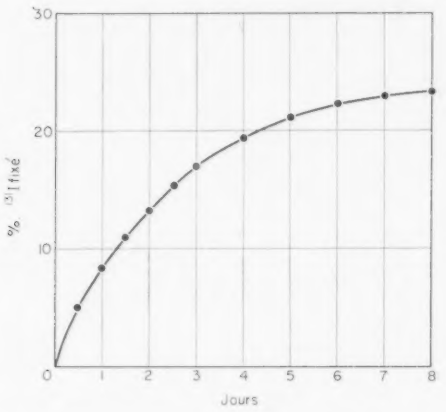


FIG. 1. Cinétique de la fixation d'$^{131}$I par les larves de *Petromyzon planeri* Bl. 20 animaux (100 g environ) immergés a +18°C, sous aération continue, dans 1500 ml d'eau additionnée de 300 $\mu$C d'$^{131}$INa sans entraîneur (activité spécifique: 5 mC/$\mu$g). L'$^{131}$I fixé a été calculé, en tenant compte de la décroissance, en pour-cent de la quantité totale présente au temps zéro. Abscisses: temps en jours; ordonnées: % d'$^{131}$I fixé.

La concentration d'$^{131}$I est particulièrement élevée dans l'endostyle et la chorde dorsale; ceux-ci renferment près de 40% d'$^{131}$I, alors que leur poids total est une fraction minime de celui de l'organisme. L'un et l'autre paraissent être les seuls à fixer les iodures, mais les proportions respectives d'$^{131}$I présentes chez eux

TABLEAU 1—RÉPARTITION DE LA RADIOACTIVÉ DANS LES DIVERSES RÉGIONS OU TISSUS DE LA LARVE DE *Petromyzon planeri* BL. APRÈS 4 ET 8 JOURS D'EXPÉRIENCE (INCUBATION À 18°C DANS DE L'EAU ADDITIONNÉE D'$^{131}$INa : 0,2 $\mu$C/ml)

| Région ou tissu | $^{131}$I fixé après (%) | |
|---|---|---|
| | 4 jours | 8 jours |
| Région de l'endostyle | 21 | 12 |
| Sérum | 2 | 1 |
| Chorde dorsale | 15 | 26 |
| Tissus mous (sans l'endostyle) | 62 | 61 |

s'inversent du quatrième au huitième jour. Il est probable que ce dernier fait reflète la diversité d'utilisation de l'halogène, l'endostyle intégrant rapidement les iodures dans des molécules organiques, tandis que tel n'est pas le cas dans la chorde dorsale (Leloup, 1952).

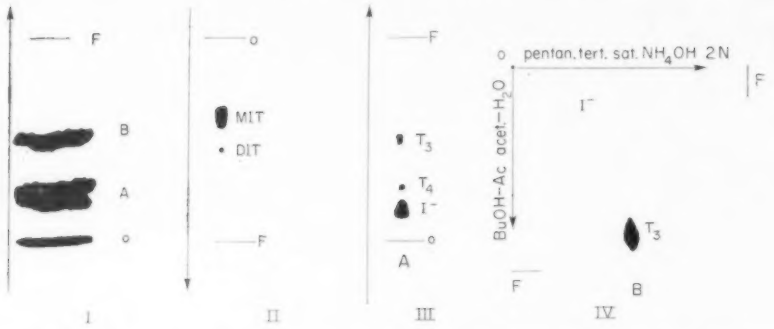


FIG. 2. Constituants iodés marqués de la région endostylaire des larves de *Petromyzon planeri* Bl., ayant séjourné 4 jours dans de l'eau additionnée d'$^{131}$INa (0,2 $\mu$C/ml).

I. Autoradiochromatogramme préparatif de l'extrait *n*-butanolique de l'hydrolysat protéinasique (solvant: pentanol tertiaire saturé d'$NH_4OH$ 2N; sens ascendant). Bandes: (O origine), A et B; F = front.

II. Autoradiochromatogramme de l'éluat de la bande O (solvant: *n*-butanol–acide acétique–eau, 78 : 5 : 17; sens descendant). O = origine; F = front; MIT = 3-monoiodotyrosine; DIT = 3 : 5-diiodotyrosine.

III. Autoradiochromatogramme de l'éluat de la bande A (solvant: pentanol tertiaire saturé d'$NH_4OH$ 2N; sens ascendant). O = origine; F = front; $I^-$ = iodures; $T_4$ = thyroxine; $T_3$ = 3 : 5 : 3′-triiodothyronine.

IV. Autoradiochromatogramme bidimensionnel de l'éluat de la bande B (solvants indiqués sur la figure). O = origine; F = front; $I^-$ = iodures; $T_4$ = 3 : 5 : 3′-triiodothyronine.

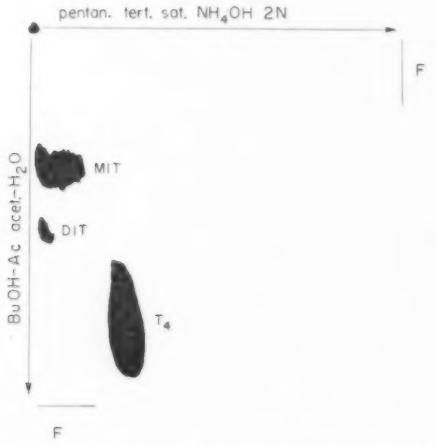


FIG. 3. Constituants iodés marqués de la région endostylaire des larves de *Petromyzon planeri* Bl. ayant séjourné 8 jours dans de l'eau additionnée d'$^{131}$INa (0,2 $\mu$C/ml).

Autoradiochromatogramme bidimensionnel de l'extrait *n*-butanolique de l'hydrolysat protéinasique (solvants indiqués sur la figure).

MIT = 3-monoiodotyrosine; DIT = 3 : 5-diiodotyrosine; $T_4$ = thyroxine.

2. *Nature des constituants iodés*

(a) *Région de l'endostyle.* L'extraction par le *n*-butanol à pH = 1 ne permettant d'extraire que des traces d'$^{131}$I des tissus broyés, les recherches ont porté sur l'hydrolysat protéinasique de ceux-ci. Environ 80% de la radioactivité sont extraits de l'hydrolysat par le *n*-butanol saturé d'HCl 0,1 N et, après élimination

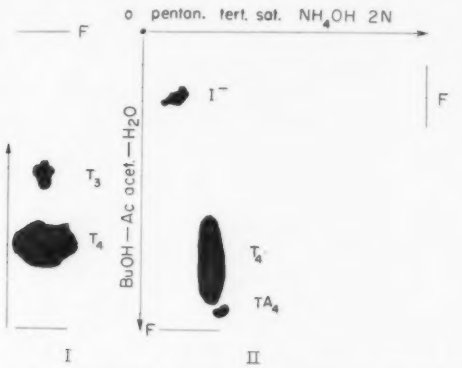


FIG. 4. Constituants iodés marqués de l'extrait *n*-butanolique des tissus mous (région de l'endostyle non comprise) des larves de *Petromyzon planeri* Bl. ayant séjourné 4 ou 8 jours dans l'eau additionnée d'$^{131}$INa (0,2 $\mu$C/ml).

I. Autoradiochromatogramme obtenu après 4 jours d'experiénce (solvant: pentanol tertiaire saturé d'$NH_4OH$ 2N; sens ascendant).

II. Autoradiochromatogramme bidimensionnel obtenu après 8 jours d'expérience (solvants indiqués sur la figure). O = origine; F = front; $I^-$ = iodures; $T_4$ = thyroxine; $TA_4$ = acide 3 : 5 : 3′ : 5′-tétraiodothyroacétique (traces).

des iodures par électrophorèse préparative sur papier, la composition du mélange des corps organiques marqués présents a été établie. Les Figs. 2 et 3 illustrent les résultats obtenus.

La région endostylaire renferme donc, au quatrième jour, deux hormones thyroïdiennes ($T_3$ et $T_4$) et leurs précurseurs (MIT et DIT), avec prédominance de MIT et de $T_3$. Au huitième jour la région endostylaire renferme $T_4$, MIT et DIT, une fraction importante de la radioactivité étant fixée à $T_4$. $T_3$ n'a pas été mise en évidence.

(b) *Tissus récepteurs.* Les tissus mous non compris dans la région de l'endostyle renferment des corps organiques iodés et des iodures; les premiers sont presque quantitativement extraits par le *n*-butanol chlorhydrique, une partie des seconds demeurant alors dans la phase aqueuse (analyse électrophorétique). L'examen de la Fig. 4 rend compte des résultats obtenus sur l'extrait *n*-butanolique.

La présence de $T_4$ et, au quatrième jour, celle de $T_3$ sont manifestes; mais, contrairement à ce que l'on observe dans la région de l'endostyle, les hormones ne sont pas accompagnées de leurs précurseurs. La répartition quantitative des constituants marqués des tissus étudiés a été mesurée, avec une approximation

satisfaisante, sur les électrophorégrammes et les chromatogrammes des divers tissus, au moyen de déterminations au chromatolecteur. Les résultats obtenus ont été rassemblés dans le Tableau 2.

TABLEAU 2—RÉPARTITION QUANTITATIVE APPROXIMATIVE (MOYENNE DÉTERMINÉE À PARTIR DE 4 LOTS DE 20 LARVES) DES COMPOSÉS IODÉS MARQUÉS PRÉSENTS DANS LA RÉGION DE L'ENDOSTYLE ET DANS L'ENSEMBLE DES AUTRES TISSUS MOUS DES LARVES DE *Petromyzon Planeri* BL. AYANT SÉJOURNÉ 4 OU 8 JOURS À +18°C DANS DE L'EAU ADDITIONNÉE D'$^{131}$INa (0,2 $\mu$C/ml)

| Composés iodés | Région de l'endostyle | | Ensemble des autres tissus mous | |
|---|---|---|---|---|
| | 4ème jour | 8ème jour | 4ème jour | 8ème jour |
| Iodures | 30 | 15 | 80 | 80 |
| 3-monoiodotyrosine | 26 | 41 | 0 | 0 |
| 3 : 5-diiodotyrosine | 2 | 10 | 0 | 0 |
| 3 : 5 : 3′-triiodothyronine | 38 | 1 | 2 | 0 |
| Thyroxine | 4 | 33 | 18 | 20 |

3. *Transport sanguin de la thyroxine*

La radioactivité du sérum peut être rapportée pour environ 50% à des iodures au quatrième jour, tandis qu'elle est à peu près entièrement fixée aux protéines au huitième jour. L'analyse chromatographique révèle alors qu'elle est presque quantitativement présente dans $T_4$. L'addition de $T_4$ au sérum des larves permet d'obtenir des autoélectrophorégrammes superposables à ceux des échantillons prélevés sur des animaux mis en expérience pendant 8 jours et dont un exemple est reproduit dans la Fig. 5.

La fixation de $T_4$ à des protéines sériques est mise en évidence par cette figure, bien que le système protéique des Cyclostomes, très différent de celui des Sélaciens ou des Téléostéens (Salvatore *et al.*, 1959) se prête moins bien à une séparation électrophorétique de ses constituants.

## DISCUSSION DES RÉSULTATS

La fixation d'$^{131}$I par les larves de *Petromyzon planeri* Bl. a permis d'étudier la fonction thyroïdienne et le métabolisme de l'iode chez celles-ci. On peut considérer comme établi par les recherches de Leloup (1955) et les nôtres que l'endostyle est le siège de l'hormonogénèse. Il renferme, en effet, les hormones tétraiodée ($T_4$) et triiodée ($T_3$), en même temps que les iodotyrosines (MIT et DIT) qui sont leurs précurseurs. MIT est toujours présente en quantité plus élevée que DIT, mais celle-ci augmente dans le temps, car le rapport MIT/DIT est égal à 19/1 au quatrième jour et à 4/1 au 8ème, la fixation des iodures du milieu étant alors pratiquement arrêtée et le rapport ne portant que sur la fraction marquée des iodotyrosines. Ces faits traduisent seulement la cinétique de la biosynthèse des hormones et de leurs précurseurs. La formation de $T_3$ prédomine dans les premiers jours où MIT n'est accompagnée que de quantités minimes de DIT, tandis que celle de $T_4$

et de DIT devient beaucoup plus importante par la suite. Ni les hormones, ni leurs précurseurs n'existent dans l'endostyle à l'état libre; ils ne peuvent en être extraits par le *n*-butanol chlorhydrique. Une hydrolyse protéinasique préalable étant nécessaire à cette extraction, on peut en inférer qu'ils sont des constituants

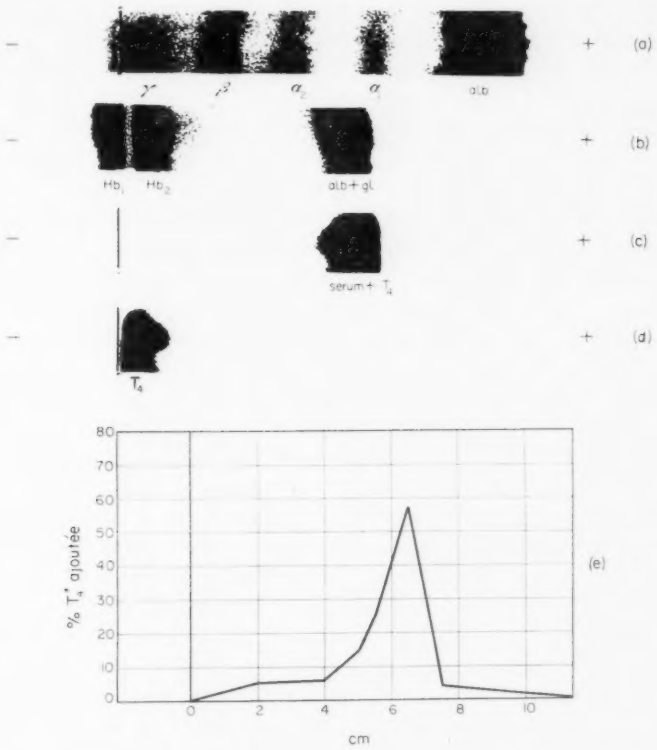


FIG. 5. Transport protéique de la thyroxine ($T_4$) marquée par $^{131}$I ajoutée au sérum des larves de *Petromyzon planeri* Bl.

(a) électrophorégramme de référence établi sur 20 $\mu$l de sérum humain.
(b) électrophorégramme de sérum de larves (faiblement hémolysé; 30 $\mu$l).
(c) autoradioélectrophorégramme du même sérum additionné de $T_4$ marquée (0,1 $\mu$g/ml).
(d) autoradioélectrophorégramme de $T_4$ marquée en solution a pH = 8,6.
(e) courbe de répartition de la radioactivité sur l'électrophorégramme du sérum de larves additionné de $T_4$.

d'une protéine analogue à la thyroglobuline, au sein de laquelle ils sont formés. Le mécanisme de l'hormonogénèse dans l'endostyle des larves de *Petromyzon planeri* Bl. paraît donc être identique à celui existant dans le corps thyroïde des Vertébrés supérieurs (Taurog & Chaikoff, 1947; Roche & Michel, 1959); il est néanmoins très sensiblement plus lent car les iodures présents dans le région de l'endostyle

constituent une fraction d'$^{131}I$ total élevée (30%) au 4ème jour et moindre, mais encore notable (15%), au 8ème.

Les tissus étrangers à la région de l'endostyle renferment une quantité importante d'iodures, même lorsque ceux-ci ne circulent plus qu'à un taux très faible dans le sérum. On y trouve $T_4$ associée à $T_3$ au quatrième jour, mais non les iodotyrosines (MIT et DIT). De plus, alors que les acides aminés iodés de l'endostyle sont compris dans une protéine, $T_4$ et $T_3$ n'existent qu'à l'état libre dans les tissus récepteurs, d'où elles peuvent être extraites quantitativement sans hydrolyse préalable par le *n*-butanol chlorhydrique. L'abondance des iodures dans les tissus récepteurs est sans doute due en partie au catabolisme cellulaire des hormones et en partie à la diffusion des ions $I^-$ circulants. Le premier de ces processus explique qu'au 4ème jour l'endostyle soit riche en $T_3$ alors que les tissus périphériques n'en renferment que des traces, $T_3$ alors que les tissus périphériques n'en renferment que des traces, $T_3$ étant alors métabolisée plus rapidement que $T_4$. De même $TA_4$, décelé à l'état de traces, est un témoin du catabolisme de $T_4$.

On peut se représenter la biosynthèse des hormones thyroïdiennes dans l'endostyle et leur utilisation cellulaire à partir des données qui viennent d'être discutées, mais les modalités de leur transport de l'endostyle aux récepteurs méritent d'être envisagées. Si l'on admet que la sécrétion de l'ébauche thyroïdienne endostylaire est holocrine (Gorbman, 1955), l'absence d'iodotyrosines (MIT et DIT) dans les tissus autres que l'endostyle ne peut s'expliquer que par l'existence d'une désiodase spécifique, analogue à celle du corps thyroïde (Roche *et al.*, 1952). Cet enzyme pourrait déshalogéner MIT et DIT après la protéolyse des cellules de l'endostyle desquamées dans le tube digestif. En effet, on ne retrouve pas d'iodotyrosines marquées dans le sang circulant où l'on a pu mettre en évidence $T_4$.

L'existence d'un transport protéique de celle-ci, comme chez les Poissons (Salvatore *et al.*, 1959) et la plupart des Vertébrés (Robbins & Rall, 1957), est compatible avec un caractère holocrine de la sécrétion, sans exclure la possibilité d'un passage direct des hormones de l'endostyle au plasma.

En dehors de l'endostyle, la chorde dorsale seule se montre susceptible de concentrer $^{131}I$, comme l'a également observé Leloup (1952). L'enrichissement de la chorde en $^{131}I$, initialement moins intense que la concentration de celui-ci par l'endostyle, se poursuit plus longtemps. Aussi, la signification de ces deux processus ne saurait-elle être identique.

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# A STUDY IN COMPARATIVE NUTRITION

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**Abstract**—(1) Experiments with representatives of thirteen phyla are presented for preliminary examination of the thesis that representative species from all phyla of life may be reared on a single syntype* diet. A more detailed examination is made of the phylum Chordata, especially in the class Mammalia. The second purpose of the work is a corollary of the thesis, "If a species cannot be reared on the diet, new principles of nutrition may be indicated".

(2) Non-fastidious microorganisms, plants and higher invertebrates survived, grew and reproduced well when fed this diet; however, difficulty was encountered in maintaining many of the lower invertebrates (sponges, coelenterates and platyhelminthes).

(3) Guppies fed the diet grew at a rate equal to that of guppies fed commercial food or a synthetic-type diet containing liver and yeast. Work with Amphibia and Reptilia was less successful and may indicate that new principles of nutrition might be learned in the feeding of frogs, turtles or alligators. The basic problem here may well be one of behaviour (appetite) rather than nutritive requirements. The diet appeared to be quite satisfactory for representatives of the class Aves: turkeys, ducks and chickens grew and feathered well and reproduction was obtained in chickens.

(4) Representatives of various orders of Mammalia were found to grow well on the diet. Opossums, dogs, kittens, monkeys, hamsters, rats, rabbits and pigs grew well and appeared healthy during the experimental period. Mice were maintained through four generations. Guinea pigs grew satisfactorily during the first generation and reproduced on the diet. To allow proper growth in lambs the diet had to be modified.

(5) The results indicate that syntype diets used for laboratory animals may be used for many other species. Diet may thus be considered a unit variable in biological research with non-fastidious organisms.

## INTRODUCTION

COMMON constituents, metabolic reactions and basic physiologic functions of all active cells indicate that certain common denominators exist in nutrition throughout the phylogenetic spectrum. As Mitchell (1945) pointed out: "The qualitative similarities among the higher animals are well established; the different species are nourished by essentially the same organic and inorganic nutrients; their ability to transform one nutrient into another is subject to essentially the same limitation so that the indispensable nutrients of a complete diet are much the same for all." An accepted principle is that the nutritive requirements of an organism can be fulfilled by approximately fifty compounds (minerals, vitamins, amino acids, fatty acids, carbohydrates and the few known and unknown factors required by fastidious organisms). A simplified concept of comparative nutrition would be obtained if

* A syntype diet is a synthetic-type diet, composed of chemically pure salts and vitamins and purified protein, fat, carbohydrate and fiber.

different species could be reared on a single diet much as blood nurtures tissues which differ in their chemical and morphologic characteristics.

Various attempts have been made to relate quantitative requirements to common denominators which would be valid for many or all animals. Crampton *et al.* (1951) found that anatomical differences between humans, rats, swine, guinea pigs and sheep do not result in large differences in digestive capacity. Sarrus & Rameaux (1939) suggest that heat production by different species is related to surface area. Brody (1945) has summarized important contributions in attempts to relate the energy requirements of different species to surface area. Minimal water requirements have also been related to surface area for different species (Richter, 1938). Interspecies requirements for endogenous nitrogen (Smuts, 1935) or vitamins (Cowgill *et al.*, 1932) are sometimes related to caloric intake. Some nutrients, as vitamin A (Guilbert *et al.*, 1940), may be required by different species according to their body weight.

Although Guilbert & Loosli (1951) attempted to state the nutritive requirements of farm animals on a generalized basis, and bacteriologists conventionally use one medium for a variety of microorganisms, no literature is found wherein an attempt was made to rear a wide variety of species from different phylogenetic classes on a single semi-synthetic diet. Many people have fed the diet made for one species with or without modification to other species. Ratcliffe (1954) indicated some success with a combination of two practical diets for many animals in the Philadelphia Zoological Gardens. Cow's milk is often used to rear the young of many species of mammals. Very little work has been directed toward determination of the nutritional requirements of the million-odd species of invertebrates. Some species have been maintained under laboratory conditions; but usually enough vestiges of the natural environment remain so that no serious attempt can be made to determine the nutritive requirements in terms of specific minerals, amino acids, carbohydrates, fatty acids, vitamins or fiber. The few studies of nutritive requirements of insects (Lipke & Fraenkel, 1956) give good evidence of a general similarity between mammalian and insect requirements.

Examination of the literature of experimental nutrition of the past decade shows striking similarity in the composition of the semi-synthetic diets used for rats, mice, chicks, dogs, monkeys, humans and even tissue cells and bacteria. They are usually 15–30 per cent protein, 40–80 per cent purified carbohydrate, 3–8 per cent fat and 2–8 per cent salts, and vitamins as needed. Such a diet was used here since the results can be interpreted better with a synthetic-type (syntype) diet than with a practical diet; completely synthetic diets were not feasible when this work began in 1952.

The purpose of this work is to determine whether the known principles of nutrition may be extended throughout the phylogenetic spectrum of living organisms. A broad basis of comparative nutrition should help to determine similarities and differences in nutritive requirements of representatives of major phyla (Luckey, 1954). Such information is needed to evaluate nutritional requirements and the inversely related biosynthetic potential of species as factors in

evolution (Loeb & Northrop, 1916). It is possible that these studies may lead to the discovery of unknown growth promotants, improved methods of feeding, or palatability factors in those species which cannot be reared with the present diet.

## METHODS

The compositions of the diets used are given in Table 1. Diet No. U-1 was designed in 1952 to be as complete as possible, with special regard to the relatively high requirement of chicks for calcium, phosphorus, manganese and protein; of guinea pigs for potassium and magnesium; of rabbits for fiber; and of dogs for fat. High quantities of vitamins were added as a precautionary measure. The sucrose was added to increase palatability. Modifications required during the 8 years will be noted. It is emphasized that this is not a synthetic diet in the sense that it contains only chemically defined ingredients. It is called a synthetic-type or *syntype* diet to indicate that certain components (as casein, starch, corn oil) are purified ingredients containing some potential for carrying unknown nutrients.

The physical character of the diet is that of a mixed powder (300–80 mesh) and was changed as needed to suit the conditions of the experiment or to appeal to the animal being fed. Most animals ate it as a powder. It was pelleted with a small amount of water for the monkeys. About 10 per cent water was mixed into it with a balling action of the fingers to make pellets which were dropped into the water for leeches, snails and turtles. Enough water was mixed into it to give the consistency of mush to feed the young dogs. Pigs, cats and lambs were fed the diet in water to give a "milk" with about 10 per cent solids. A more dilute mixture (about 2 per cent solids) was fed to chameleons, sponges, tadpoles, microorganisms and vinegar eels. It was fed as bits of a thin wafer (10 per cent water was mixed in, then it was dried on glass) to fish and planaria. All were fed *ad libitum* unless otherwise specified in the results section. Tap water was constantly available to the vertebrates, and distilled water was used for all other species.

Most animals were maintained in a laboratory without air-conditioning. The "land" animals, except mice, were maintained in cages with wire mesh floor. With the exception of *Turbitrix aceti* which kept its environment clean, the species reared in non-sterile aquaria were cleaned routinely a short time after feeding each day.

The systematic approach outlined for this work was an attempt to rear one representative species for each major phylum of living organisms. Poor availability of suitable material and inability to maintain some of the species have limited the thoroughness but not the scope of this report. An attempt was made to rear a representative of each phylum listed in Table 2, one representative of each class listed under the phylum Chordata, and one or more representative of each order listed under the class Mammalia. A five-fold increase in live weight and a clear indication of health was considered to be the minimum criterion of success; maximum growth rate was not considered *the* criterion of success. A more rigorous criterion was reproduction. Some of the vertebrates and invertebrates were carried through one or more generations, while the microorganisms were taken through

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many generations with five to ten transfers. The numbers of individuals reared was ten or more for at least one species in each phylum, but was as few as two for many of the mammalian species. Two was considered an adequate number of

TABLE 1—COMPOSITION OF DIETS

| Ingredients (g/kg) | Diet number | | |
|---|---|---|---|
| | U-1 | U-2 | U-3 |
| Casein, Labco | 300 | 300 | 300 |
| Glucose | | | 480 |
| Starch, corn | 300 | 200 | |
| Alpha cell | 120 | 60 | 30 |
| Cellophane spangles | | 60 | 30 |
| Sucrose | 120 | 120 | |
| Oil, corn | 80 | 80 | 80 |
| $Na_2MoO_4 \cdot {}_2H_2O$ | | 0·30 | 0·30 |
| Vitamin A, I.U. | 10,000 | * | * |
| Vitamin D, I.U. | 2000 | | |
| Vitamin E | 0·30 | | |
| Vitamin K (Menadione) | 0·010 | | |
| Vitamin C | 10 | | |
| Thiamin | 0·020 | | |
| Riboflavin | 0·020 | | |
| Pyridoxine | 0·020 | | |
| Ca pantothenate | 0·050 | | |
| Niacin | 0·100 | | |
| Biotin | 0·001 | | |
| Folic acid | 0·020 | | |
| Choline Cl | 2 | | |
| Vitamin $B_{12}$ | 0·0005 | | |
| *i*-Inositol | 2 | | |
| Starch, corn to (Vit. carrier) | 5 | | |
| Salts L–II | 60 | | |

* Same as diet U-1 for all ingredients below this mark.

| | | |
|---|---|---|
| | $CaCO_3$ | 18·0 |
| | $CaHPO_4$ | 3·3 |
| | $K_2HPO_4$ | 13·5 |
| | $NaHPO_4$ | 12·0 |
| | NaCl | 3·0 |
| | KI | 0·045 |
| | $MgSO_4 \cdot 7H_2O$ | 4·5 |
| | $MnSO_4 \cdot 4H_2O$ | 0·75 |
| | $Fe(C_6H_5O_7)_2$ | 4·5 |
| | $CuSO_4 \cdot 5H_2O$ | 0·23 |
| | $CoCl_2 \cdot 6H_2O$ | 0·03 |
| | $ZnSO_4 \cdot 7H_2O$ | 0·06 |
| | $Na_2B_4O_7 \cdot 10H_2O$ | 0·03 |
| | $AlK(SO_4)_2 \cdot 12H_2O$ | 0·045 |
| MgO | | 4 |
| K acetate | | 20 |

animals to determine qualitatively that members of that species would grow and survive with apparent health when fed the diet under the conditions specified.

TABLE 2—ORGANISMS FED A SINGLE DIET 1952–1959

| Phyla | Genus | Species | Common name |
|---|---|---|---|
| Schizophyta | *Escherichia* | *coli* | |
| | *Lactobacillus* | *arabinosis* | |
| Eumycophata | *Penicillium* | *expansum* | |
| | *Saccharomycetes* | *cerevisiae* | |
| Algata | *Chlorella* | *sp.* | |
| Spermatophyta | *Lycopersicon* | *esculentum* | tomato |
| | *Lemna* | *minor* | duckweed |
| Protozoa | *Tetrahymena* | *geleii* | |
| Porifera | *Spongilla* | *fragilis* | sponge |
| Coelenterata | *Hydra* | *oligactis* | hydra |
| Platyhelminthes | *Planaria* | *sp.* | planaria |
| Nemathelminthes | *Turbotrix* | *aceti* | vinegar eel |
| Annelida | *Placobdella* | *parasitica* | leech |
| Mollusca | *Helisoma* | *companulata* | snail |
| Arthropoda | *Armadillidium* | *vulgare* | pill bug |
| | *Liobunum* | *vittatum* | spider |
| | *Blattella* | *germanica* | cockroach |
| | *Acheta* | *domesticus* | cricket |
| Chordata | | | |
| Class | | | |
| Pisces | *Carassius* | *auratus* | goldfish |
| | *Lebistes* | *sp.* | guppy |
| Amphibia | *Rana* | *pipiens* | grass frog |
| Reptilia | *Kinosternon* | *odoratum* | turtle |
| | *Pseudemys* | *elegams* | turtle |
| | *Alligator* | *mississippiensis* | alligator |
| | *Anolis* | *carolinensis* | chemaleon |
| Aves | *Anas* | *platyrhynchos* | Pekin duck |
| | *Gallus* | *domesticus* | White Leghorn chick |
| | *Meleagris* | *gallopavo* | Beltsville white turkey |
| Mammalia | | | |
| Order | | | |
| Marsupialia | *Delephis* | *virginiana* | opossum |
| Carnivora | *Canis* | *familiaris* | dog |
| | *Felis* | *domesticus* | cat |
| Primate | *Macaca* | *rhesus* | monkey |
| Rodentia | *Mus* | *musculus* | mouse |
| | *Rattus* | *norvegicus* | rat |
| | *Cavis* | *cobaya* | guinea pig |
| | *Cricetus* | *cricetus* | hamster |
| Lagomorpha | *Lepus* | *cuniculus* | domestic rabbit |
| Artiodactyla | *Sus* | *scrofa* | pig |
| | *Capra* | *hircus* | kid |
| | *Ovis* | *aries* | lamb |

Weights were taken at weekly intervals. The fish were weighed in a tared beaker of water. The length of the guppies was obtained by placing them in a small quantity of water in a Petri dish which was placed over graph paper with

mm rulings. Food efficiency data were taken near the end of some experiments. Results are cited as individual experiments which were not necessarily performed in chronological sequence.

## RESULTS

### Phylum SCHIZOPHYTA

Results with microorganism are given in Table 3. The phylum Schizophyta was examined with one Gram-negative and one Gram-positive organism. A pure culture of the Gram-negative bacteria *Escherichia coli* grew very well when washed

TABLE 3—RESULTS WITH MICROORGANISMS

| Phyla and organism | No. of transfers | Media | Age (days) | Growth |
|---|---|---|---|---|
| Schizophyta | | | | |
| *Escherichia coli* | 10 | Liquid | 2 | 55 mil/ml |
| *Lactobacillus arabinosis* | 10 | Liquid | 2 | 25 mil/ml |
| Eumycophyta | | | | |
| *Penicillium expansum* | 10 | Agar-like | 5 | Covered surface |
| *Saccharomyces cerevisiae* | 11 | Liquid | 2 | Good |
| Algata | | | | |
| *Chlorella sp.* | 5 | Liquid | 2 | Good |
| Protozoa | | | | |
| *Tetrahymena gelleii* | 10 | Liquid | 3 | Good |

in saline and inoculated into about 10 ml of a 2 per cent mixture of the diet in water. One loop of inoculum was used. After 24 hr a fresh tube was inoculated from the first 10 ml culture. The growth in the tenth consecutive tube determined by plate dilution methods at 48 hr was 55 million individuals per millilitre of media. The abundant growth indicated this was a satisfactory food for this organism which actually required relatively few of the ingredients (Peterson & Peterson, 1945).

When the more fastidious bacterium, *Lactobacillus arabinosis*, was grown in pure culture in a 2 per cent mixture of the diet in water, it reproduced almost at the same rate as had *E. coli*. 25 million organisms per millilitre were found in the tenth serial transfer 2 days after inoculation. This representative of Gram-positive organisms needs the majority of compounds in the diet, and the qualitative or quantitative excesses do no real harm.

### Phylum EUMYCOPHATA

One loop of a *Penicillium expansum* was inoculated into 2 per cent diet in water in a test tube. Good growth was visible at 4 days, the surface appeared green at 6 days and darkened during the next few days. *Penicillium lanosum* was grown in a

Petri plate on a more concentrated (about 16 per cent) mix of the diet in water. It also grew luxuriantly. Ten serial transfers were made of 4-day-old cultures which indicated that this representative of the molds could be grown indefinitely.

A representative of the yeasts, *Saccharomyces cerevisiae*, was grown on a broth made of 2 per cent of the diet in water. It reproduced rapidly as evidenced by turbidity and was transferred every 48 hr for ten transfers. The growth was verified by transferring the culture to Sabarouds agar medium.

Phylum ALGATA

Three plants were reared with the diet. A pure culture of *Chlorella sp.* was placed in an Erlenmeyer flask containing 10 ml of 2·5 per cent diet in water. Two loopfuls of organisms were transferred to a new flask after 2–3 days of growth. Vigorous growth occurred through the fifth transfer. Then all flasks were maintained under a 100 W bulb for about 3 weeks. One of the five was contaminated by mold, and the others appeared healthy with every indication that they could be maintained indefinitely.

Phylum SPERMATOPHYTA

The duckweed, *Lemna minor*, was reared aseptically through five subcultures on the surface of a dilute solution of the diet in water. Reproduction during the

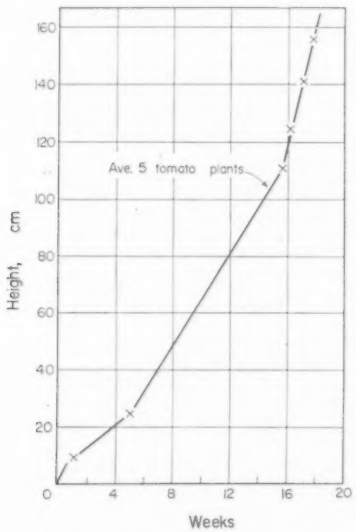


FIG. 1. Spermatophyta *Lycopersicon esculentum.*

2-month experiment resulted in a fifty-fold increase in total number of plants. Tomato seeds were planted in rows perpendicular to rows of the diet mixed in sand. This arrangement provided different concentrations of fertilizer for different

seeds. They were placed in a south window and watered daily. The growth rate (height) was improved when more diet was mixed into the sand a few centimeters from the sturdiest plants and artificial light was used (Fig. 1). The plants continued to grow until the experiment ended at 20 weeks. No flowers or fruit were seen, but the leaves and stems showed good color throughout the experiment.

Phylum PROTOZOA

The protozoan, *Tetrahymena geleii*, was grown in pure culture in 1 per cent diet No. U-1 in water. The pH of the mixture was 6·2 after heat sterilization. Each culture was subcultured every 3 days for ten consecutive transfers. Although no counts were taken, growth and reproduction of the protozoa were vigorous, indicating that the culture could be continued indefinitely.

Phyla PORIFERA, COELENTERATA and PLATYHELMINTHES

Attempts to rear representatives of the phyla Porifera and Coelenterata indicated the need for further study. The sponge gemmules were developing slowly when this experiment was stopped. The hydra never seemed to form a healthy colony in the presence of the diet. These and planaria may need to be reared in lower concentration of minerals.

Phylum NEMATHELMINTHES

*Turbotrix aceti* could not be maintained in a sterile solution of the diet in water. It was adapted to the diet by successive transfers into solutions with decreasing concentrations of vinegar and greater proportions of the diet. The species was thereafter grown and allowed to reproduce in a 2 per cent mixture of the diet in water for five successive transfers. Two loopfuls of the solution which contained about fifteen eelworms were placed in 10 ml of fresh solution. These increased in number in a week to approximately 2000 when the next subculture was made.

Phylum ANNELIDA

*Placobdella parasitica* were maintained for over 2 months in an aquarium in which they were fed moistened diet No. U-1 balled with the fingers into a 1 cm mass. The leeches were quickly attracted to the diet and ate it. This observation was confirmed by the fact that those fed the diet were much fatter and survived longer than others which received no food. Growth was not precisely measured in this species, but the individuals fed the diet appeared to be in good health.

Phylum MOLLUSCA

Fifteen specimens of *Helisoma companulata* were grown for 158 days in an aquarium with small pellets of moistened diet No. U-1 being fed each day. They were 2 months old at the start of the experiment. Eggs were seen 34 days after the experiment began and these eggs were allowed to hatch 38 days later by moving the snails to a new jar for a week. Twelve of the second generation were maintained until the experiment was terminated 172 days later. Two of the first and one of the

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second generation died during the experiment. Since the wet and dry weights of the fleshy body could be correlated to the increased size of the shell, growth was recorded as the maximum diameter of the shell. The composite growth curve (Fig. 2) indicated that the snails responded well to the diet in this environment.

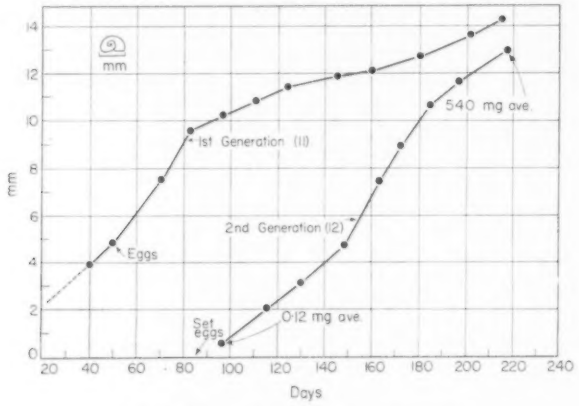


FIG. 2. Mollusca *Helisoma companulata*.

Newly hatched snails average 0·87 mm in length and 0·125 mg in weight. The body *sans* shell varies from 40–55 per cent of the total weight; the average of forty snails of different ages during the experiment was 51 per cent. Since the ratio of shell diameter to total weight is 1·10 in the early growth period and 1·50 later, the body weight increment during the experiment was more than 1000 times the starting weight. Snails previously grown on syntype diets (Howes, 1937) gained ten times their starting weight in 17 weeks while the second generation reported herein gained in the same period 4500 times their hatch weight.

Phylum ARTHROPODA

*Armadillidium vulgare* will eat the diet in powdered form quite readily, but growth over an extended period has not yet been obtained due to the high humidity requirements of this species. This representative of the class Crustacea was maintained for 1 month.

No success has been attained in any attempts to rear members of the class Arachnida. They have not been induced to eat the diet.

Representatives of the class Insecta eat the diet readily. Three successive generations of *Blattella germanica* were reared when fed powdered diet No. U-1 and water. The growth rate (Fig. 3) and reproduction are comparable to those obtained earlier (House, 1949). *Acheta domesticus* grew at a low rate (ave. 4·3 mg at 4 weeks) when fed the syntype diet. They grew faster (ave. 70 mg at 4 weeks) when fed only powdered rye. The greatly increased rate of growth (ave. 151 mg

at 4 weeks) obtained when the rye and the syntype diet were fed together (50–50 mix by volume) indicated two nutritional problems: (a) rye contains an unknown

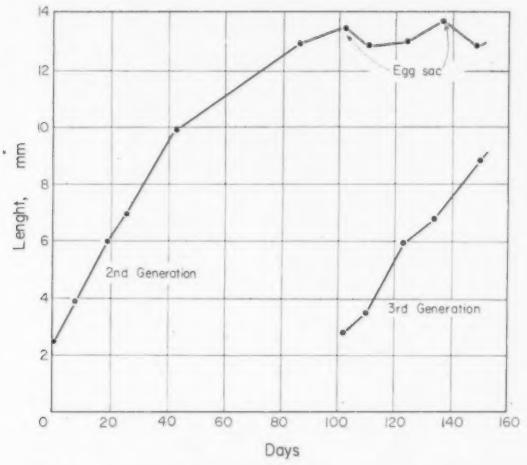


FIG. 3. Arthropoda—Insecta *Blattella germanica.*

nutrient needed by the cricket which was not in the syntype diet, and (b) the diet supplies a nutrient which is low or missing in the rye grass. Both problems are being explored.

## Phylum CHORDATA

### *Pisces*

Two goldfish were fed the diet in wafer form for a period of 20 weeks. The length of both fish increased three-fold during the experiment (3·3 to 9·6 cm and 2·3 to 8·3 cm respectively).

Results from one experiment with guppies is presented in Fig. 4. The fish fed diet U-1 grew as well as did control fish fed the commercially prepared diet plus live algae or a second control group fed the syntype diet plus 50 per cent whole liver powder. The survival of the algae in the control tank required that a 200 W light bulb be placed about a foot above the water. These growth rates were comparable to those of salmon fed a syntype diet by Halver *et al.* (1957). The individual variations in length of the guppies were usually within 5 per cent of the total length. After 3 months the fish appeared healthy and were growing satisfactorily so the experiment was discontinued.

### *Amphibia*

Young tadpoles (about 4 days old) were obtained commercially and fed by sprinkling the powdered diet on the water. The variation in length of different individuals was very marked (about two-fold) while the variation in weight

deviated only ±5 per cent from the average. Growth and development were consistent (Fig. 5) for 6 weeks. One developed precociously; its rear legs developed slowly after the first 6 weeks. The tails decreased somewhat in size but body growth

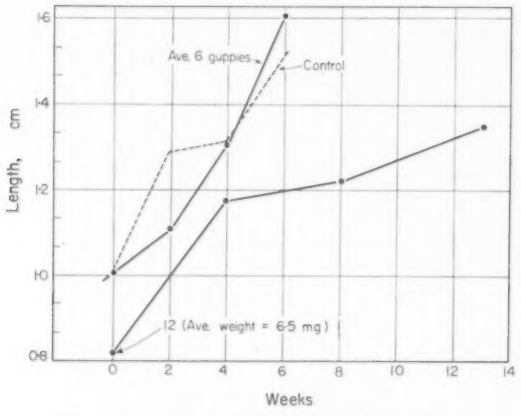


FIG. 4. Chordata—Osteichthyes *Lebistes sp.*

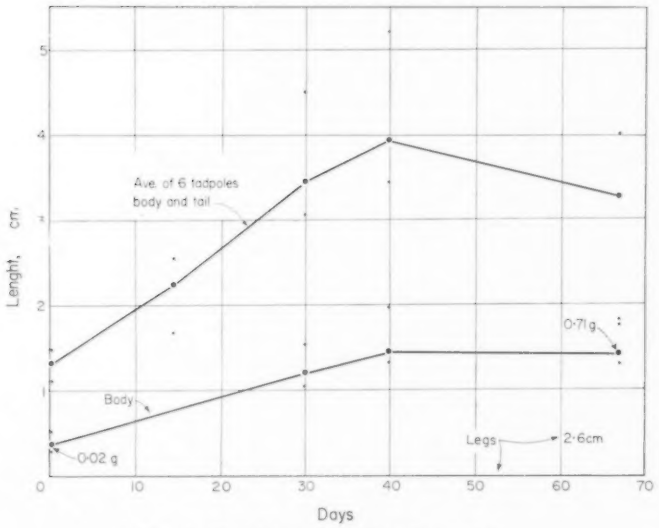


FIG. 5. Chordata—Amphibia *Rana pipiens.*

was poor and leg development was slow. Administration of iodinated casein to some of them increased the speed of development of the legs but did not materially increase growth rate. Transfers to a balanced aquarium seemed to speed both growth and development in the retarded amphibians.

*Aves*

Diet No. U-1 was fed to Beltsville White turkeys, Pekin ducks and White Leghorn chicks. The five turkeys ate well and developed normally. Ducks grew satisfactorily on the diet as illustrated by the growth curve (Fig. 6). The growth was faster than that reported by Miller (1953). The diet adhered to the inner edge of the beak of chicks so that the mass prevented effective eating. When starch was

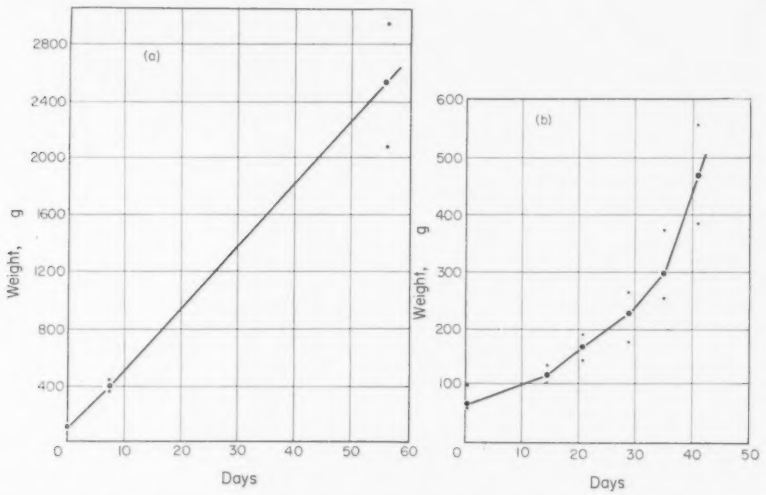


FIG. 6. Chordata—Aves. (a) *Anas platyrhynchos*. Two Pekin ducks. (*b*) *Gallus domesticus*. Six White Leghorn chicks.

substituted for sucrose (giving a total of 42 per cent starch in the diet) the gumminess decreased and the chicks grew satisfactorily over a 6-week period (Fig. 6). Good body and feather development were obtained with turkeys, ducks and chicks. The birds show little pigmentation of the beak and legs when fed this diet which contains little carotenoid pigments.

In a subsequent experiment seven of ten chicks were continued to maturity. They weighed $1204 \pm 75$ g at 3 months and began laying vigorously at 7 months. Unfortunately a diet change invalidated the experiment after only four eggs were obtained. One of the four eggs hatched and the second generation chick appeared healthy. This confirms the work of Jukes & Sokstad (1946) and of Reyniers *et al.* (1949) who reared chicks through reproduction on a syntype diet containing natural sources of vitamins.

*Reptilia*

One small turtle (a 607 g *Kinosternon sp.* obtained from Hemlock Lake in Michigan) readily ate small moist pellets of the diet dropped into the water near it. Unfortunately he was lost and other turtles obtained commercially could not be induced to eat the diet.

Three chameleons (weight 2–4 g and length 13–17 cm) were induced to eat by allowing a dilute mixture of the diet in water run down the side of the cage. They would lick up the moving droplets whereas they ignored it otherwise. They were maintained for 4 months when the experiment was discontinued. None grew materially in weight or total length during that period.

Baby alligators failed to grow when fed U-1 as a milk via stomach tube. Autopsy revealed a large gall bladder as the only gross anomaly. This indicated that starvation was a contributing factor to death. When more than the usual amount (ca. 5 ml) of food was given, regurgitation became a problem. Neville (1958) has eliminated force-feeding problems by a technique that gives promise of success in experimental reptile nutrition. The new method is simply to put the diet in capsules which are dipped in corn oil and placed in the water. These are readily eaten (usually swallowed whole) by the alligators. The capsule modified the diet equivalent to the addition of 20 per cent gelatin.



*Mammalia*

*Marsupialia.* Three opossums ate the diet readily and grew rapidly. The fur grew long, the skin was soft, the eyes were bright and they all became fat. Two had

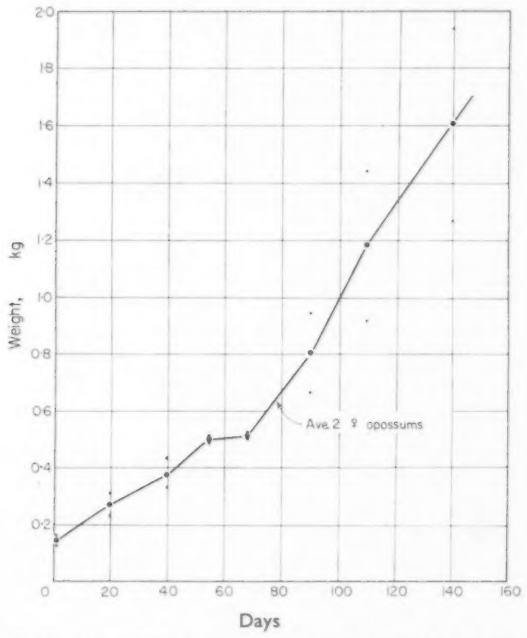


FIG. 7. Chordata—Mammalia—Marsupialia *Didelphis virginiana.*

a progressive necrosis of the tail which started with a scaliness at the tip. In one it progressed for about 2 in., the tip came off and the stump healed nicely within a

month. In the other the "wet ring disease" continued to the end of the experiment 2 months later. One (female) weighed 547 g when obtained and grew to 1327 g in 4 months. The growth rate for the other two is presented in Fig. 7. The experiments were terminated when the two females averaged ten times their initial weight.

TABLE 4—FOOD EFFICIENCY STUDIES

| Genus | Duration (days) | Food | | | Gain | | Food efficiency (%) |
|---|---|---|---|---|---|---|---|
| | | Total (g) | Ave./day (g) | Wt. (kg) | Total (g) | Ave./day (g) | |
| *Didelphis* | 6 | 342 | 57 | 0·415 | 9 | 1·5 | 2·6 |
| | 5 | 249 | 50 | 0·650 | 112 | 22·4 | 44·9 |
| | 6 | 379 | 63 | 0·762 | 213 | 35·5 | 56·2 |
| | 7 | 574 | 82 | 0·975 | 415 | 59·3 | 72·2 |
| *Canis* | 5½ | 1703 | 310 | 6·45 | 530 | 96·4 | 31·3 |
| *Ovis** | 7 | 2401 | 343 | 9·45 | 850 | 121 | 35·3 |
| | 7 | 2942 | 420 | 10·30 | 1250 | 179 | 42·6 |
| *Lepus* | 4 | 381 | 95 | 1·97 | 158 | 39·5 | 41·6 |
| | 4 | 296 | 74 | 1·64 | 99 | 24·8 | 33·5 |
| *Cavia* (3 pooled) | 3 | 386 | 43 | 1·37 | 78·3 | 8·7 | 20·2 |

* Average of pooled data from two lambs. The two sets of data were taken on successive weeks.

The food efficiency of these animals varied greatly. When the data were obtained from one (Table 4) she was growing very little. Possibly the extra attention upset her. The data taken from the other were obtained during the period of maximum growth rate. Water consumption was higher than was expected and larger water jars were given to it at the beginning of the second and third determination. This suggests that the high value of 72 per cent food efficiency may have been partially saturation of tissues with water despite the fact that the food consumption was higher during this period than on other days.

*Canis.* A 2-month-old mongrel pup was fed Diet U-1 mixed in milk for 4 days, after which the diet was mixed with an equal weight of water to a pudding-like consistency. She ate the diet readily, grew at a rate of ½ kg/week (Fig. 8), had luxuriant fur and tremendous vitality. Five months after the termination of the experiment one of her 5-week-old puppies was obtained for the second experiment. It ate readily, grew well and appeared healthy. The growth of this mongrel puppy was somewhat greater than that reported by Ontko *et al.* (1957) for the growing dog fed a practical diet. The food efficiency (Table 4) was 31 per cent.

*Felis.* Two kittens from a litter of five were started on experiment at 10 days of age. They were healthy and grew as well as litter mates maintained in private homes (Fig. 9). The average weekly gain of 154 g per week compared favorably with the values given by Miller & Allison (1958) of 135 and 160 g gain per week in female and male kittens respectively and is better than that reported by Gershoff *et al.* (1957).

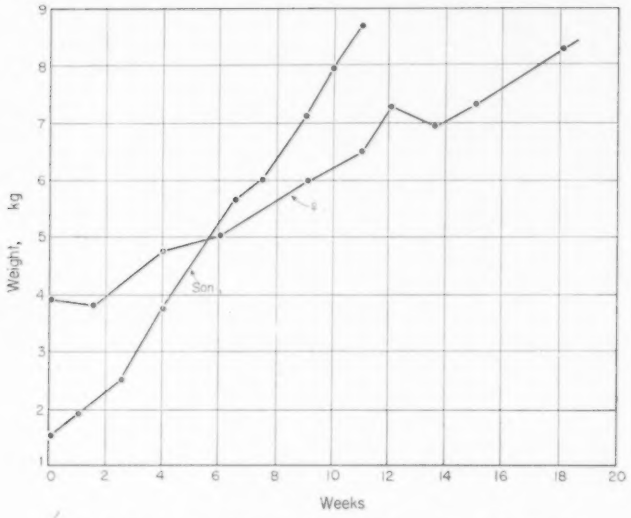


FIG. 8. Chordata—Mammalia—Carnivora *Canis familiaris*.

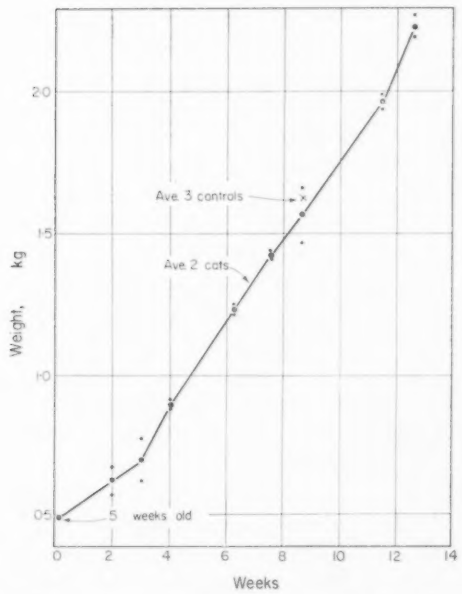


FIG. 9. Chordata—Mammalia—Carnivora *Felis domesticus*.

*Macaca.* Three monkeys were fed Diet U-1 as a slightly moistened ball. After 1 month, the diet of the heaviest was changed to a control diet (fruit, vegetables, bread and a commercial dog food). The diet change caused considerable weight loss which was regained within 3 weeks. The other two were maintained on the syntype diet for 5 months, at the end of this period their average weight was

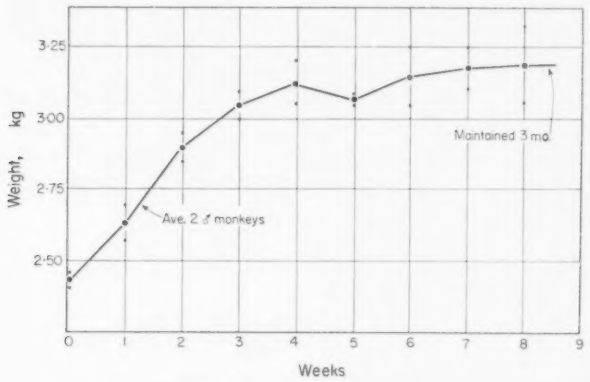


FIG. 10. Chordata—Mammalia—Primate *Macaca rhesus.*

2·0 kg (1·85 and 2·15 kg) and the control monkey weight 2·0 kg. The general health of the monkeys was good throughout the experimental period. Their stools were about the same consistency as that of other monkeys in this colony, usually somewhat pasty, sometimes loose and sometimes formed. The diet was quite adequate for maintenance. The growth pattern (Fig. 10) was similar to that reported by Waisman *et al.* (1943) for this species.

*Rodentia.* One pregnant mouse was fed Diet U-1 through parturition and lactation. Six of the young (first generation) were fed only this diet and water from 3 weeks of age until they had reproduced and reared the second generation through weaning. Some of the mice from the second generation were continued on the diet (mice from two litters reared in a common jar with sawdust for nesting) until they had reproduced and weaned third generation mice. When six of these had grown to maturity, reproduced and weaned the fourth generation mice the experiment was discontinued. The growth curves of the mice (Fig. 11) show that each successive generation had a higher average weight at weaning and possibly a greater growth rate than the mice of the previous generation.

Reproduction and lactation were comparable to that in the stock colony fed commercial diet. Mice of the first generation began to reproduce at 74 days (birth to parturition date). Only one mouse survived to weaning in the first three litters. Thereafter attempts to obtain birth weights were stopped, and three of the four female mice reared a litter to weaning before they were discarded. Mice in the second generation began casting litters at 65 days of age. First litters were weaned

by all four females. Third generation mice were born of mothers beginning when they were 60 days old. All four females weaned their first litter. Records were not made of the number of mice in each litter. The mice appeared normal and healthy

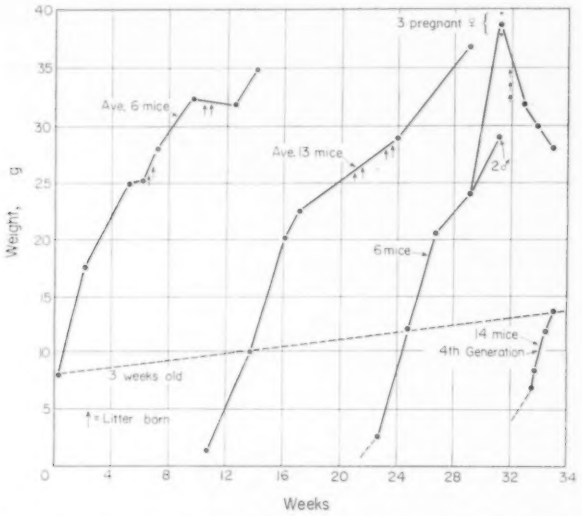


FIG. 11. Chordata—Mammalia—Rodentia *Mus musculus* (white).

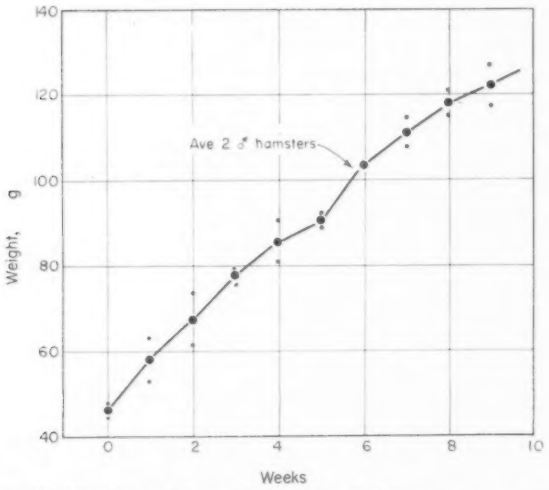


FIG. 12. Chordata—Mammalia—Rodentia *Mesocricetus auratus*.

throughout the four generations. The growth generally was better than that reported previously (Luckey *et al.*, 1955) for three generations of mice fed a syntype diet which contained yeast and liver.

Two young male hamsters were fed diet U-1 and water for 9 weeks. Their growth (Fig. 12) and general appearance were good throughout the experiment. The average weekly gain of 9 g is comparable to that obtained by Ershoff (1956).

Five weanling, Wistar-strain, white rats (male) were fed Diet U-1 for 7 weeks. They appeared to be healthy and their growth rate (Fig. 13) average 27 g per week. This is comparable to results from others (Richardson & Brock, 1958) using similar diets.

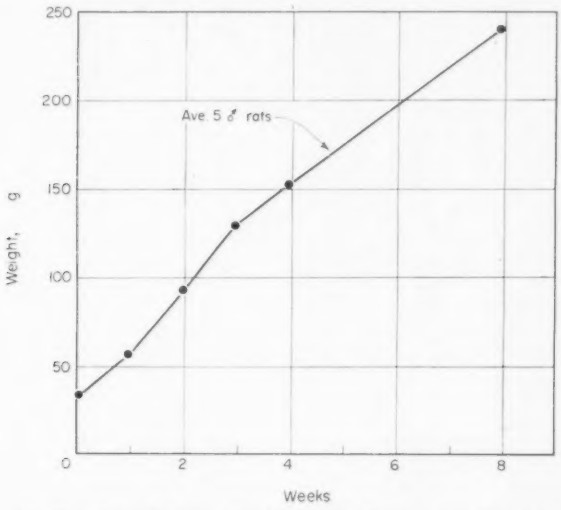


FIG. 13. Chordata—Mammalia—Rodentia *Rattus norvegicus* (white).

Three guinea pigs were placed on experiment when 1 week old. They ate the diet readily and appeared equal in health and vigor to guinea pigs reared on stock diets. The first generation pigs grew an average of 3 g per day during the first 20 weeks and ate an average of 43 g per day when their average weight was 456 g. The weight decrease in the male at 5 months coincided with the onset of breeding activity. Three young were born to one of the females when she was 9 months old. The young appeared to be healthy, suckled quite heavily and were completely weaned to the syntype diet plus water at the age of 1 week. The second generation guinea pigs averaged $4\frac{1}{2}$ g gain per day (Fig. 14) for the 14 weeks they were on experiment. Throughout the experiment they were alert, clean, well furred and appeared to be in excellent health. These results in growth and reproduction are comparable to those reported by Reid & Briggs (1953) with a syntype diet.

*Lagomorpha.* Two weaned domestic rabbits were fed Diet U-1 in dry form for a period of 18 weeks. They ate well, grew satisfactorily (Fig. 15) and appeared to be well furred and quite healthy. Similar weight gains were obtained by Hove & Herndon (1957) with rabbits fed purified diets. Food consumption, taken at 5

weeks when the rabbits were gaining 25 and 50 g per day, was 74 and 95 g per day. The food efficiency for these two was 33·5 and 41·6 per cent respectively. Autopsy

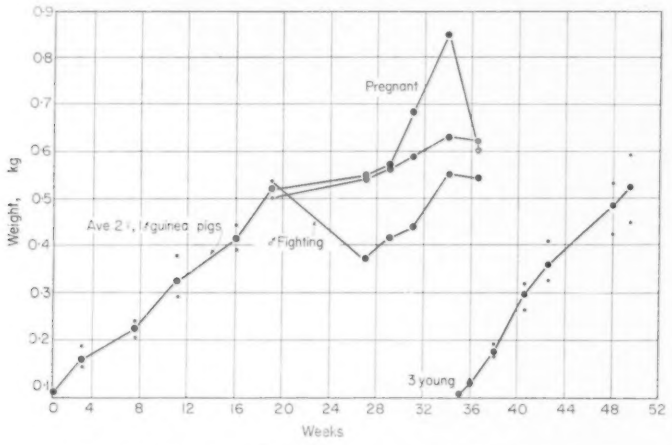


FIG. 14. Chordata—Mammalia—Rodentia *Cavia cobaya*.

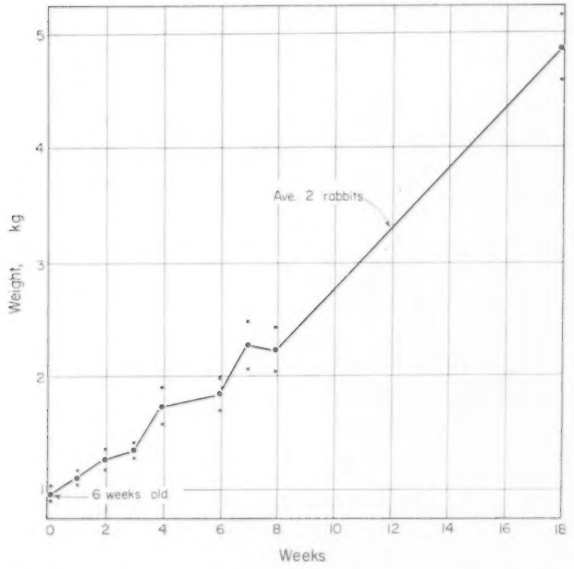


FIG. 15. Chordata—Mammalia—Logomorpha *Lepus cuniculus*.

at 24 weeks revealed no gross abnormalities; both presented fat healthy carcasses. Reproduction was obtained in another experiment with rabbits fed a similar syntype diet.

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*Artiodactyla.* Two Hampshire pigs taken from the sow at 1 week were fed Diet U-1 as a thick milk. After 2 days of bottle feeding they learned to eat without being bottle fed and ate a wet mash of the diet *ad libitum*. They appeared quite healthy and strong. Their stools were very dark green and well formed. The growth averaged 248 g per day, a somewhat faster rate than the litter mates maintained on the farm. This rate of gain is about twice that obtained by Miller *et al.* (1957). The experiment was concluded when body weight had increased ten-fold.

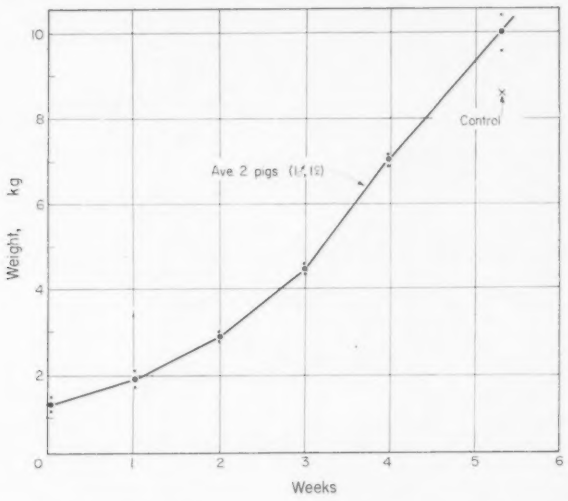


FIG. 16. Chordata—Mammalia—Artiodactyla *Sus scrofa* (Hampshire).

Two young lambs were maintained for 3 and 5 weeks respectively. Both grew well and both died after a brief sickness in which digestive disturbance was suspected from the lack of regular feces and the distended abdomen. Autopsy revealed impaction; the fiber (alpha-cell) of the diet was not being passed from the stomach. It formed a hard plug at the pyloric sphincter which apparently stopped passage of both solid and fluid material into the intestine.

For the next experiment two 4-day-old lambs of a Hampshire–Shropshire cross were purchased locally. After 1 week the male ate poorly. Since impaction was apparent, the diet was changed by adding 6 per cent cellophane spangles at the expense of the alpha-cell (Diet U-2). It responded favorably to sulfa drugs and antibiotics. At 1 month both lambs began to nibble at solid food, in addition to eating about 150 g of solids in the milk daily. Their growth (Fig. 17) was good. At 2 months the male began to eat sporadically, had much coughing and died. The experiment was discontinued. Their growth (Fig. 17) averaged 210 g per day for the first 7 weeks. Excreta were generally soft and light orange. Autopsy of the female revealed no abnormalities in blood clotting time or gross morphology. Its

rumen was well developed (about the size of the head). The omasum and abomasum were not well differentiated. All linings of the gastro-intestinal tract appeared to be healthy. The male had congestion in one small lobe of the lung. The heart, kidney, brain, liver, muscles, intestine, fat and fascia appeared normal. All four stomachs showed degeneration, being about twice the size of those of the female lamb. The linings, particularly the maniplies, were poorly developed, flabby, and contained a dark watery mass quite different from the gray fluid in the rumen of the female.

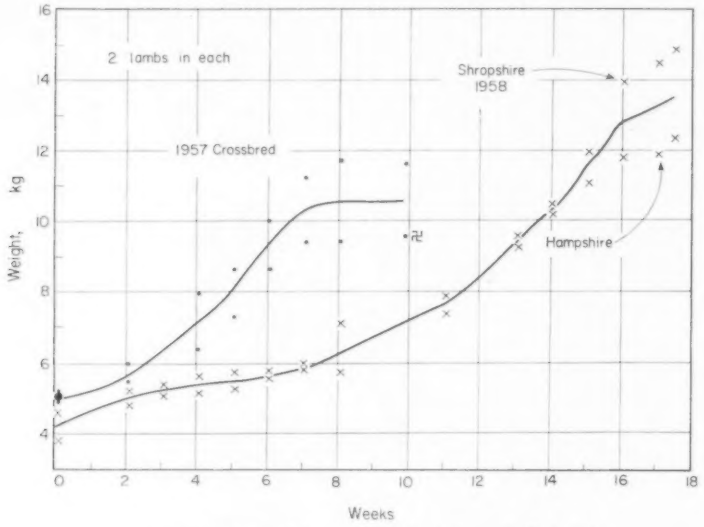


FIG. 17. Chordata—Mammalia—Artiodactyla *Ovis aries*.

In the next experiment two 4-day-old male lambs, one Hampshire and one Shropshire, were fed Diet U-3 as a "milk" with water and solid food available *ad libitum*. They were fed by bottle four times daily during the first month, three times daily for 2 weeks and twice daily thereafter. It is noted they were apparently not getting enough food in the milk form since they grew poorly (Fig. 17) until they began to eat solid food in the third month.

Diet U-3 contained only 6 per cent fiber, and glucose replaced both sucrose and starch. The latter was made in accordance with the finding of Porter (1957). The fiber was cut from 12 per cent to 6 per cent at 7 weeks and they grew well beginning at 8 weeks. They appeared to be very healthy, more energetic than any lambs in previous experiments. They would often jump sideways or kick up their feet during the short exercise, a 60 m run twice daily. The increased vitality was probably due to the change in carbohydrate. At 3 months one of the lambs began pulling the wool from the other which became denuded on one rear leg and a good portion of that side and shoulder. This stopped after the fourth month and

new wool began to grow. The feces generally were well formed black pellets after the first month. The increase in wither height of the Shropshire was 35·6–47·0 cm and that of the Hampshire was 33·6–44·4 cm during the 18-week experiment. The average food consumption of the lambs at $3\frac{1}{2}$ months was 420 g per day, and the food efficiency was about 39 per cent (Table 4). The average gain for a 10-week period from the time the fiber content was reduced to 6 per cent until the end of the experiment was 150 g per day. This compared well with both the results of Matrone *et al.* (1957) and the value for 24 lb per 5 months (73 g per day) reported for Shropshire lambs by Brody (1945).

The general appearance of the lambs was good. The wool was well formed and heavy. Autopsy revealed some consolidation in the lungs, about 1/20th was non-functioning. Other organs appeared to be normal. The rumen had about four liters of viscous dark gray fluid which smelled similar to rumen contents of grass-fed cattle. Leaflets of mesenteric fat were present, but no large fat deposits were noted in either of these lambs.

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## DISCUSSION

The results give only a first approximation of the actual requirements of any species reared. Such a simplified concept gives no information comparable to that known about nutritive requirement of the rat, mouse or chick. The specific qualitative and quantitative requirement of each species, if desired, could be determined by manipulating the individual ingredients of the diet. The diet contains an excess of many of the known micronutrients. For some of the species reared a given nutrient may not be present in adequate amounts for the greatest growth rate; other species may not need that particular compound at all. Any excess must necessarily be relatively innocuous if the diet is to be of general use. For this diet no ingredient was present in toxic quantities, and enough nutrients were present to sustain life, growth and well-being of most species. A long list of compounds which might be added for fastidious organisms would indicate one limitation of this particular diet. The addition of these would make the application of the diet more universal. The lower level of roughage would be preferable for the non-herbivores. In these respects and in the fact that it is not a synthetic diet this work should be considered as a guide to future studies. Since it is similar to many diets used in recent years, it provides a measuring device.

The data indicate the validity of the thesis: nutritional principles learned with usual laboratory species are applicable generally. Diets developed for mammals and birds are useful for rearing species in other phyla. Species from ten different phyla will live and grow when fed this diet. Closer experimental examination of Chordata indicated that greater success was obtained in this phylum than might be expected from the invertebrate phyla. However, success with members of the invertebrate phyla may be more meaningful in the sense that there are many more invertebrate species. Detailed examination indicates that most mammalian species could be reared on this diet. The results with Amphibia and Reptilia suggest that unknown nutritional factors may be needed by these animals. The difficulties may

be due to either appetite or unsuitable environment rather than new diet constituents. Ready acceptance of the diet by most species verified the suggestion that strict carnivores can be reared without meat (Luckey & Hittson, 1952), and natural eating habits need not be observed when feeding young animals. The improved performance of successive generations of mice is of considerable interest.

The use of a single standard diet may in one sense eliminate nutrition as a variable in comparative biology studies. This study illustrates that gross morphological changes are not induced by diet. Minor changes in morphology have shown to be effected by diet such as vitamin or mineral deficiencies or the addition of small quantities of antibiotics (reviewed by Luckey, 1959). Defense mechanisms are affected by diet (Schneider, 1956; Axelrod, 1958). Quantitative nutritive requirements change markedly with variation of dietary ingredients (Luckey *et al.*, 1946). Many physiologic activities are impaired in malnutrition. Enzyme activity in tissues varies with dietary constituents (Ross, 1956). The presence of carotenoid pigments in diet directly effects the carotenoid deposition in the tissues. Comparative biochemistry is therefore directly affected by diet.

The use of a single diet is one step in the standardization of biological material. Uniformity of diet, light, temperature, humidity, barometric pressure, air flow, dust, gas composition, water and noise level are possible. Acceptable standards should be formulated and used when interlaboratory comparisons are made and standard values are needed. Control of microbic contamination and genetic make-up are variables which could be controlled by using germ-free techniques and identical sibs (as in *Dasypus*).

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# A COMPARISON OF THE EFFECT OF LONG DAILY PHOTOPERIODS ON THE PATTERN OF ENERGY STORAGE IN MIGRATORY AND NON-MIGRATORY FINCHES*

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**Abstract**—Caged white-crowned sparrows, *Zonotrichia leucophrys gambelii*, when subjected to long daily photoperiods in mid-winter, deposit large amounts of subcutaneous and visceral fat and develop migratory behavior comparable to natural vernal migratory behavior. The altered metabolic state involved in this deposition of fat is characterized by a reduction in glycogen content of the pectoral muscles and liver and a suppression of the diurnal glycogen cycle in these organs. There is also a marked increase in the fat content of these tissues so that the total mobilizable energy in them is considerably greater than in the short-day winter birds. The thigh muscles show a similar reduction in glycogen content but only a very slight increase in fat content. This change in the pattern of energy storage may be regarded as one feature of the scheme which produces the metabolic alterations necessary for sustained migratory flight.

The wintering population of the Oregon Junco, *Junco oreganus montanus*, in southeastern Washington responds, although less extensively, to long daily photoperiods. This may be correlated with its more restricted migratory movements. The non-migratory English sparrow, *Passer domesticus*, when subjected to long daily photoperiods, shows no such alteration in the pattern of energy storage in pectoral muscle and liver; rather there is a tendency toward a lower level of energy storage.

Although *Zonotrichia leucophrys gambelii* in mid-winter have much less glycogen in pectoral muscle and liver than the indoor caged birds, the pattern of energy storage may be consistent with that of the latter in experimentally induced migratory state if the restricted flight activity of the latter, the precapture activity of the former, and possible differences in temporal pattern of food intake are taken into consideration.

The relatively small amount of glycogen available, and the extensive storage of fat, suggest that there must be very extensive use of fat or fatty acids directly by the pectoral muscles during flight.

## I. INTRODUCTION

IT has long been known that many, if not most, migratory birds deposit large quantities of fat before and during the migratory periods (Wachs, 1926; Zedlitz,

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1926; Wolfson, 1945; Farner, 1955; Merkel, 1958). Although premigratory fattening is usually associated with both migratory periods, our attention here will be restricted to the vernal fat deposition. Obviously fat deposition requires that the bird be in a state of positive energy balance. Such a state may be attained by increased energy intake or by reduction of energy expenditure, or by a combination of both. A reduction in energy expenditure could be effected by a reduction in the rate of some component(s) of the so-called basal or resting metabolism (Wachs, 1926; Groebbels, 1928, 1930, 1932) or of some component(s) of the non-basal metabolism. The latter could involve then a shift in the utilization of surplus energy in the sense of Kendeigh (Kendeigh, 1949; Seibert, 1949; Wallgren, 1954). Apparently it was Zedlitz (1926) who first suggested that premigratory deposition of fat is primarily the result of an increased energy intake. This suggestion has been confirmed subsequently for the vernal premigratory fat deposition in *Erithacus rubecula* (Merkel, 1958), *Zonotrichia albicollis* (Odum & Major, 1956; Weise, 1956), *Zonotrichia leucophrys gambelii* (King & Farner, 1956; King, 1957), *Spizella arborea* (Weise, 1956), *Emberiza hortulana* (Wallgren, 1954), *Fringilla coelebs* (Koch & de Bont, 1952) and probably *Fringilla montifringilla* (Rautenberg, 1957). In the case of *Emberiza hortulana*, Wallgren (1954) has suggested that the elimination of the afternoon period of activity during the actual migratory period has a significant energy-sparing effect. Since this change in activity pattern appears to be common during migration (Palmgren, 1944; Farner, 1955; Farner *et al.*, 1954; Eyster, 1954; Weise, 1956; Merkel, 1958; etc.), it is possible that Wallgren's suggestion may be widely applicable.

Whatever the basis for fattening may be, there is now adequate evidence that it is photoperiodically induced by the lengthening days of spring in several species, including *Junco oreganus* (Wolfson, 1942, 1945), *Junco hyemalis* (Wolfson, 1954), *Zonotrichia albicollis* (Odum & Major, 1956), *Zonotrichia leucophrys leucophrys* (Wolfson, 1954), *Zonotrichia leucophrys gambelii* (Farner & Mewaldt, 1955; King & Farner, 1956), *Fringilla montifringilla* (Schildmacher & Steubing, 1952) and *Fringilla coelebs* (de Bont, 1947). In the case of *Zonotrichia leucophrys gambelii* the vernal premigratory fattening is caused by a photoperiodically induced hyperphagia so that the earlier suggestions of energy-sparing changes in basal metabolism (Wachs, 1926; Groebbels, 1928, 1930, 1932) are not applicable at least in this case (King & Farner, 1956; King, 1957).

It is obvious that a period of positive energy balance and fat deposition must involve profound metabolic changes. The occurrence of afternoon respiratory quotients in excess of 1·0 during this period in several species is an indication of the synthesis of fat from carbohydrate and protein (Merkel, 1958).

The function of this paper is to describe changes in the lipid and glycogen content of the skeletal muscles and liver of *Zonotrichia leucophrys gambelii* during the periods of photoperiodically induced hyperphagia and fattening, both natural and experimental. Comparisons are made with *Passer domesticus*, which is non-migratory, and with a local population of *Junco oreganus montanus*, in which migratory behavior is not as extensively developed as in *Zonotrichia leucophrys*

*gambelii*. The photoperiodically induced changes in storage of fat and glycogen in the liver were first observed histochemically and noted very briefly in conjunction with an investigation of photoperiodically induced changes in hypothalamic neurosecretion (Oksche *et al.*, 1959). Because we now place greater importance on these changes, and because of the importance of localization of them, the histochemical observations will be presented in greater detail here.

## II. MATERIALS AND METHODS

The white-crowned sparrows (*Zonotrichia leucophrys gambelii*) and juncos (*Junco oreganus montanus*) used in these investigations were captured with Japanese mist nets from wintering flocks in the Snake River Canyon of southeastern Washington and from migrating flocks in the immediate vicinity of Pullman, Washington. English sparrows (*Passer domesticus*) were netted or trapped on the campus of the Washington State University. Reserve stocks of captive birds were maintained in large outdoor aviaries. These, as well as experimental birds, were fed a nutritionally adequate chick-starter mash having fat, protein and carbohydrate components of 3·1 per cent, 20·7 per cent and 74·7 per cent respectively, plus mineral and vitamin additives. The caloric content was 4·4–4·5 kcal/g dry weight. This feed, and water as well, were available *ad libitum*. For photoperiodic experiments the birds were placed, two per cage, in Hendryx breeding cages ($22 \times 41 \times 26$ cm) after being held in the large outdoor aviaries for at least a week. Birds held continuously in the outdoor aviaries have weight and gonadal cycles in winter and spring which are almost identical with those of wild birds (Farner & Wilson, 1957). All birds were weighed once per week or more frequently. In experiments with altered daily photoperiod, all groups received light from incandescent lamps adjusted to give a uniform illumination of about 600 lux in the middle of the cage. This is far above the maximal intensity for photoperiodic responses with long continuous photoperiods in these species. The lengths of the photoperiods were controlled by automatic switches. The indoor aviary temperatures were maintained at approximately 22°C.

All birds used for chemical estimation of glycogen and lipid content of liver and muscle were killed by decapitation immediately after capture or removal from cage. Liver and pectoral muscles were removed immediately and placed in 30% KOH for digestion or frozen on dry ice for analysis later. Samples of these tissues were also collected and frozen for fat extraction. Liver samples (mid- and distal portions of lobes) were in the range of 200–400 mg fresh weight; pectoral muscle (removed always from the same area midway between the keel and lateral margin of the muscle mass) were ordinarily within the range of 700–900 mg. The entire mass of thigh muscle was removed for estimations of fat and glycogen in this tissue. Glycogen was estimated by the anthrone method of Peterson & Rose (1951). In estimation of fat content the tissue was first weighed, then dried for 24 hr in a vacuum desiccator, weighed again, subjected to Soxhlet extraction with diethyl ether for 24 hr, dried again, and weighed. The dry weight obtained in this procedure was used to calculate percentage of water in the tissue which, in turn,

was used in the calculation of glycogen content on dry-weight basis from the original data which were on wet-weight basis. Since the quantity of energy available in carbohydrate and fat, respectively, is of primary importance, the data have been converted to kilocalories per gramme of tissue dry weight for presentation in Figs. 1, 2 and 3. For purposes of conversions we have used 4·2 kcal per g for glycogen and 9·5 kcal per g for fat.

For histochemical studies pieces of liver were removed and placed in Rossman's fluid at 0°C within 10 sec after decapitation. After washing in 96% ethanol and treatment successively with 100% ethanol and cedar-wood oil, they were embedded in paraffin. The 6-micron sections were first oxidized in 1% aqueous periodic acid (McManus & Cason, 1950) or in the 1% aqueous lead tetra-acetate mixture of Shimizu & Kumamoto (1952). They were then treated with Schiff reagent prepared from basic fuchsin treated with sodium bisulfite. Slides with sections from birds subjected to different experimental conditions and killed at different times of the day (Table 6) were placed in the same rack in order to assure uniform treatment. Control sections from each tissue sample were treated with saliva (30 min) or with a 1% solution of highly purified diastase (Merck, Darmstadt) in 1% NaCl (1 hr). Nuclei were stained lightly with hematoxylin or hemalum.

## III. THE PHOTOPERIODIC EXPERIMENTS

Many of the experimental and field data were obtained in conjunction with investigations on the annual cycle of hypothalamic neurosecretion and the role of daily photoperiod therein. Some additional data were obtained from control groups in experiments on the relation between osmotic stress and photoperiodically induced testicular development. Thus the procurement of some of the data has involved compromises in schedule which were not necessarily ideal from the aspect of the investigation reported in this paper. However, in each case they do contribute to a more complete picture of the relationship between the duration of the daily photoperiod and the energy reserves of liver and pectoral muscle and have been included for this reason.

### 1. *Zonotrichia leucophrys gambelii*

*Groups Zl-1, Zl-2 and Zl-3.* (Groups *Zonotrichia* long-day 1, *Zonotrichia* long-day 2 and *Zonotrichia* long-day 3. See Table 1.) Groups Zl-1 and Zl-2 contained fifteen and nine adults, respectively, and were subjected to twenty-three consecutive 20-hr daily photoperiods beginning on 24 and 30 December, respectively. On the twenty-fourth day the birds were killed at selected intervals throughout the day. Group Zl-3 contained four adults subjected to 20-hr daily photoperiods, two for 13 days beginning on 8 December and two for 15 days beginning 15 January. The changes in body weight are recorded in Table 1, together with rates of testicular growth, the latter as an indication of the degree of photoperiodic stimulation. The testicular development in these two groups, as indicated by testicular weights and testicular growth-rate constants (Table 1), is very similar to that attained

naturally during the migratory period (Farner & Wilson, 1957). The increases in body weight (Table 1) are reasonable indicators of the amount of fat deposited in subcutaneous and visceral depots (McGreal & Farner, 1956; King & Farner,

TABLE 1—RÉSUMÉ OF PHOTOPERIODIC EXPERIMENTS

| Species | Group | No. of birds | Daily photo-period (hr) | Experi-mental periods | No. of days | Mean change in body wt. (g) | No. of males | Mean final testes wt. (mg) | Mean* *k* |
|---|---|---|---|---|---|---|---|---|---|
| *Zonotrichia leucophrys gambelii* | *Z*s-1 | 10 | 8 | Nov. | 7 | +0·6 | 10 | 0·8 | 0 |
| | *Z*s-2 | 9 | 8 | Dec. | 7 | −1·3 | 9 | 0·4 | 0 |
| | *Z*s-3 | 4 | 8 | Dec.–Feb. | 14–19 | −1·4 | 4 | 0·8 | 0 |
| | *Z*s-4 | 8 | 8 | Dec.–Jan. | 22, 23 | −0·1 | 4 | 0·5 | 0 |
| | *Z*m-1 | 12 | 13 | Dec.–Feb. | 75 | +2·7 | 11 | 42·1 | 0·024 |
| | *Z*m-2 | 11 | 13 | Mar.–Apr. | 30 | +6·5 | 10 | 31·9 | 0·053 |
| | *Z*l-1 | 15 | 20 | Dec.–Jan. | 23 | +5·4 | 13 | 118·7 | 0·098 |
| | *Z*l-2 | 9 | 20 | Dec.–Jan. | 23 | +4·4 | 6 | 105·8 | 0·092 |
| | *Z*l-3 | 4 | 20 | Dec.–Feb. | 13, 15 | +2·6 | 4 | 23·1 | 0·102 |
| | *Z*l-4 | 8 | 20 | Dec.–Jan. | 22, 23 | +4·4 | 5 | 69·6 | 0·096 |
| *Junco oreganus montanus* | *J*s-1 | 11 | 8 | Dec. | 9 | −1·6 | 6 | 0·4 | |
| | *J*s-2 | 9 | 8 | Feb. | 8–9 | −1·7 | 7 | 1·5 | |
| | *J*l-1 | 12 | 20 | Dec.–Jan. | 27 | +1·3 | 8 | 72·0 | |
| | *J*l-2 | 4 | 20 | Jan.–Feb. | 27 | +1·4 | 4 | 140·0 | |
| | *J*l-3 | 15 | 20 | Feb.–Mar. | 27 | +1·0 | 11 | 116·8 | |
| *Passer domesticus* | *P*s-1 | 12 | 8 | Dec. | 9 | −3·7 | 5 | 1·4 | |
| | *P*s-2 | 12 | 8 | Jan. | 12 | −0·2 | 9 | 20·3† | |
| | *P*l-1 | 12 | 20 | Jan.–Feb. | 20 | −1·0 | 8 | 198·0 | |
| | *P*l-2 | 25 | 20 | Feb. | 20–21 | −3·7 | 11 | 220·7 | |

* This is a comparison of testicular growth rates on the basis of the testicular logarithmic growth-rate constant *k* (Farner & Wilson, 1957). The differences among groups *Z*l-1, *Z*l-2, *Z*l-3 and *Z*l-4 are not significant. The difference between *Z*m-1 and *Z*m-2 is a reflection of the change in the sensitivity of the testicular photoperiodic response mechanism between early December and late March. The comparison of the photoperiodic responses of the groups of *Junco oreganus montanus* and *Passer domesticus* on the basis of testicular growth rates is not possible because of lack of sufficient information on the resting testis weight and the functional relationships with time and the duration of the daily photoperiod.

† Natural vernal development of the testes had begun before this group was placed on 8-hr daily photoperiods.

1959). Previous studies (King & Farner, 1956) have shown that birds, photoperiodically treated as in groups *Z*l-1, *Z*l-2, *Z*l-3, *Z*l-4, and having attained similar increases in body weight, are invariably in migratory state as indicated by the development of *Zugunruhe*. Since no significant sexual differences in the deposition of fat and glycogen could be detected, the data for the two sexes have been combined in all experimental groups. Furthermore there were no apparent significant differences among *Z*l-1, *Z*l-2 and *Z*l-3; consequently the data from these three

groups have been combined for presentation in Fig. 1. For comparative purposes the mean glycogen and fat estimations during a period extending from 3 hr before the end of the daily photoperiod to 1 hr after the end of the photoperiod have been assembled in Tables 2 and 3.

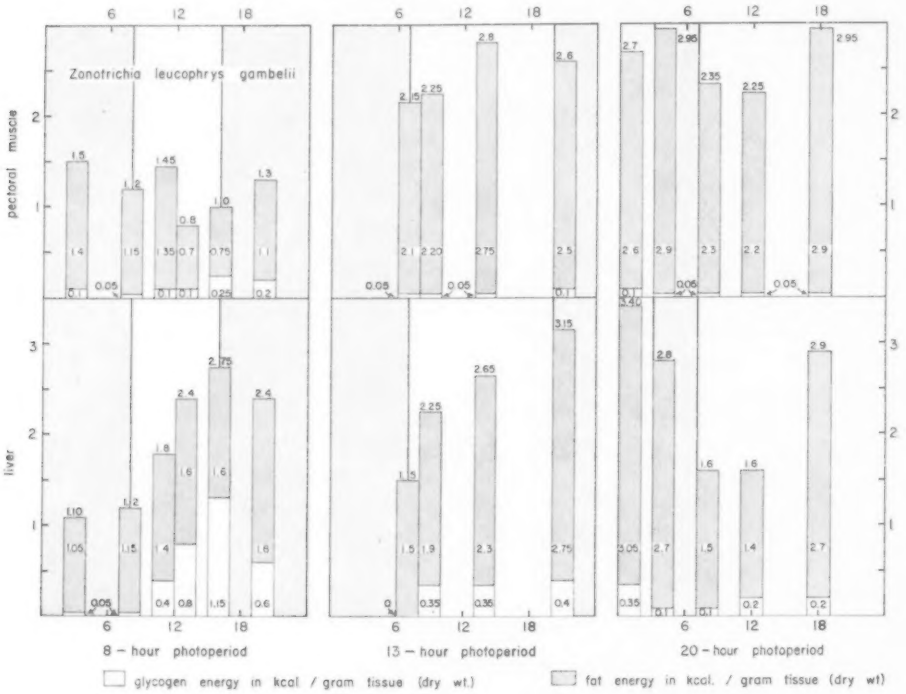
Zonotrichia leucophrys gambelii
pectoral muscle
liver
8 – hour photoperiod
13 – hour photoperiod
20 – hour photoperiod
glycogen energy in kcal / gram tissue (dry wt.)
fat energy in kcal. / gram tissue (dry wt)

FIG. 1. *Zonotrichia leucophrys gambelii.* Energy reserves in pectoral muscle and liver in relation to daily photoperiod, mean values. No. of birds: 8 hr, 24; 13 hr, 23; 20 hr, 34.

*Groups Zs-1, Zs-2 and Zs-3.* (Groups *Zonotrichia* short-day 1, *Zonotrichia* short-day 2 and *Zonotrichia* short-day 3. See Table 1.) Groups Zs-1 and Zs-2 consisted of ten and nine adults, respectively; these were held for 8 days on 8-hr photoperiods after transfer from outdoor cages (Table 1) on 23 November and 24 December, respectively. These serve as short-day controls for groups Zl-1, Zl-2, Zl-3, Zm-1 and Zm-2 (Table 1). The schedule for killing and the presentation of the data are essentially the same as for groups Zl-1, Zl-2 and Zl-3.

*Groups Zl-4 and Zs-4.* These were also 20-hr experimental and 8-hr control groups, respectively (Table 1). However, in this case both groups were subjected to the same experimental lighting scheme beginning 30 December (four in each group) or 20 January (four in each group) and continuing for 23 days. The data have been included primarily for comparison of the responses of pectoral and thigh muscles to long daily photoperiods. All birds were killed at the end of the

photoperiod on the twenty-third day in order to have all data on body weight, glycogen and fat on a strictly comparable basis. These data are recorded in Tables 1 and 4. Birds in *Z*l-4 were in migratory state at the end of the experiment.

*Groups Zm-*1 *and Zm-*2. (Groups *Zonotrichia* medium-day 1 and *Zonotrichia* medium-day 2. See Table 1.) These were 13-hr experimental groups. Group *Z*m-1 consisted of twelve first-year birds subjected to 13-hr daily photoperiods beginning on 3 December and continuing for 75 days. Group *Z*m-2 contained eleven first-year birds, similarly treated for 30 days beginning 25 March. The shorter period in spring is because of the greater sensitivity of the photoperiodic response mechanism at this time (Laws, Farner, Kamemoto, unpublished). The data for these two groups have been combined in Fig. 2 since there appears to be no significant difference between them. The presentation of the data (Fig. 1, Tables 1, 2, 3) is the same as that for groups *Z*l-1 and *Z*l-2. Previous investigations (Farner & Mewaldt, 1955; Farner, 1959) have shown that birds subjected to such photoperiodic treatment and with similar increases in body weight are invariably in migratory state as indicated by the development of *Zugunruhe*. The testicular development attained in groups *Z*m-1 and *Z*m-2 is similar to that which is attained during the early part of the migratory period (Farner & Wilson, 1957). It should be noted here that the photoperiodic responses of first-year birds are similar although not strictly identical with those of adults (Farner & Mewaldt, 1955; Farner & Wilson, 1957).

The data presented in Fig. 1 and Tables 2, 3 and 4 indicate clearly that photoperiodic stimulation with 13-hr or 20-hr daily photoperiods causes a distinct increase in the fat content of liver and pectoral muscle and a decrease in glycogen content in these organs. There is also an increase in visceral and subcutaneous fat as indicated by the increases in body weight (Tables 1, 2, 3, 4). Obviously photoperiodic stimulation causes a marked suppression of the diurnal glycogen cycle (Fig. 1). The behavior of the thigh muscles (Table 4) is of interest in that the increase in fat is relatively slight.

2. *Junco oreganus montanus*

*Groups Jl-*1, *Jl-*2 *and Jl-*3. (Groups *Junco* long-day 1, *Junco* long-day 2 and *Junco* long-day 3. See Table 1.) These groups contained thirty-four birds in adult plumage. These were subjected to 20-hr photoperiods daily for 27 days beginning 16 December, 24 January and 15 February, respectively, as indicated in Table 1. On the twenty-eighth day they were killed at selected intervals. The mean changes in body weights are recorded in Table 1 together with testicular weights as an indication of the degree of photoperiodic stimulation. The estimations of glycogen and fat in liver and pectoral muscle are presented in Fig. 2. In this figure and in Table 2 the data from these groups have been combined since there appear to be no significant differences among them. For comparative purposes the mean glycogen and fat estimations for birds killed during the period extending from 3 hr before the end of the photoperiod until 1 hr after the beginning of the dark period are recorded in Table 2.

TABLE 2—A COMPARISON OF THE CONCENTRATION OF GLYCOGEN AND FAT IN THE LIVER AND MUSCLE AT END OF THE DAILY PHOTOPERIOD. EXPERIMENTAL AND WILD GROUPS. *Zonotrichia leucophrys gambelii**

| Groups | Description | No. of birds | Body wt. (g) | | | Liver glycogen (mg/g dry wt.) | Liver fat (mg/g dry wt.) | Muscle glycogen (mg/g dry wt.) | Muscle fat (mg/g dry wt.) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | Final | Difference | | | | |
| Zs-1, Zs-2, Zs-3, Zs-4 | Indoor cages, ca. 22°, 8-hr daily photoperiods | 13 | 24·4 ± 0·53† | 25·2 ± 0·49† | + 0·8 ± 0·29† | 219·9 ± 21·1† | 168·4 ± 18·4† | 36·7 ± 7·6† | 83·6 ± 10·1† |
| Zm-1, Zm-2 | Indoor cages, ca. 22°, 13-hr daily photoperiods | 7 | 27·7 ± 0·07 | 32·3 ± 1·45 | + 4·5 ± 1·21 | 99·0 ± 8·2 | 293·2 ± 23·2 | 24·7 ± 3·0 | 272·6 ± 21·5 |
| Zl-1, Zl-2, Zl-3, Zl-4 | Indoor cages, ca. 22°, 20-hr daily photoperiods | 9 | 24·3 ± 0·94 | 32·1 ± 1·68 | + 7·8 ± 1·03 | 73·3 ± 9·7 | 306·1 ± 23·6 | 18·6 ± 3·2 | 292·6 ± 39·8 |
| Zww | Wild, midwinter, −1° to +5°, ca. 8·5–11-hr daily photoperiods. Jan.–Feb. 1958 | 16 | | 26·7 ± 0·40 | | 7·5 ± 2·2 | 130·1 ± 6·0 | 2·0 ± 0·4 | 68·1 ± 3·9 |
| Zwp | Wild, premigratory, ca. 10°, ca. 14-hr daily photoperiods. 17 April 1958 | 9 | | 28·1 ± 0·67 | | 16·0 ± 6·2 | 259·2 ± 24·2 | 6·9 ± 2·4 | 101·2 ± 7·6 |
| Zwm | Wild, migratory, ca. 10°, ca. 14·5-hr daily photoperiods. 25 April 1958 | 8 | | 29·2 ± 0·87 | | 4·4 ± 3·6 | 241·9 ± 17·2 | 2·2 ± 0·7 | 87·2 ± 5·0 |

* See Table 1 for further details. † Standard Error.

TABLE 3—A COMPARISON OF THE EFFECT OF LONG DAILY PHOTOPERIODS ON THE CONCENTRATION OF GLYCOGEN AND FAT IN LIVER AND PECTORAL MUSCLE AT THE END OF 8 AND 20 hr DAILY PHOTOPERIODS IN THREE SPECIES*

| | Groups | Duration of daily photoperiod (hr) | No. of birds | Body wt. (g) | | | Liver glycogen (mg/g dry wt.) | Liver fat (mg/g dry wt.) | Muscle glycogen (mg/g dry wt.) | Muscle fat (mg/g dry wt.) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Initial | Final | Difference | | | | |
| *Zonotrichia leucophrys gambelii* | Zs-1, Zs-2, Zs-3, Zs-4 | 8 | 13 | 24·4 ± 0·53† | 25·2 ± 0·49† | + 0·8 ± 0·29† | 219·9 ± 21·1† | 168·4 ± 18·4† | 36·7 ± 7·6† | 83·6 ± 10·1† |
| | Zm-1, Zm-2 | 13 | 7 | 27·7 ± 0·07 | 32·3 ± 1·45 | + 4·5 ± 1·21 | 99·0 ± 8·2 | 293·2 ± 23·2 | 24·7 ± 3·0 | 272·6 ± 21·5 |
| | Zl-1, Zl-2, Zl-3, Zl-4 | 20 | 9 | 24·3 ± 0·94 | 32·1 ± 1·68 | + 7·8 ± 1·03 | 73·3 ± 9·7 | 306·1 ± 24·6 | 18·6 ± 3·2 | 292·6 ± 39·8 |
| *Junco oreganus montanus* | Js-1, Js-2 | 8 | 10 | 18·6 ± 0·47 | 17·4 ± 0·38 | − 1·2 ± 0·26 | 125·4 ± 15·5 | 265·2 ± 13·9 | 35·8 ± 6·6 | 101·6 ± 10·4 |
| | Jl-1, Jl-2 | 20 | 10 | 17·5 ± 1·34 | 18·6 ± 1·38 | + 1·2 ± 1·19 | 87·7 ± 13·5 | 199·2 ± 21·3 | 13·6 ± 2·1 | 186·3 ± 27·5 |
| *Passer domesticus* | Ps-1 | 8 | 6 | 30·1 ± 0·53 | 27·4 ± 0·64 | − 2·7 ± 0·38 | 34·8 ± 10·6 | 288·8 ± 25·8 | 39·9 ± 9·7 | 193·1 ± 47·3 |
| | Ps-2 | 8 | 5 | 25·9 ± 0·23 | 25·6 ± 0·40 | − 0·3 ± 1·25 | 66·5 ± 28·3 | 322·6 ± 33·2 | 37·9 ± 7·0 | 72·1 ± 11·4 |
| | Pl-1, Pl-2 | 20 | 12 | 28·1 ± 0·57 | 24·5 ± 0·64 | − 3·6 ± 0·58 | 88·5 ± 23·6 | 177·9 ± 27·3 | 27·3 ± 3·9 | 96·4 ± 25·0 |

* See Table 1 for further details. † Standard Error.

TABLE 4—A COMPARISON OF THE CONCENTRATION OF GLYCOGEN AND FAT IN PECTORAL AND THIGH MUSCLES AT THE END OF 8 AND 20 hr DAILY PHOTOPERIODS. *Zonotrichia leucophrys gambelii**

| Groups | Duration of daily photoperiod (hr) | No. of birds | Body wt. (g) | | | Pectoral muscle glycogen (mg/g dry wt.) | Pectoral muscle fat (mg/g dry wt.) | Thigh muscle glycogen (mg/g dry wt.) | Thigh muscle fat (mg/g dry wt.) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | Final | Difference | | | | |
| Zs-4 | 8 | 8 | 27·7 ± 0·64† | 27·4 ± 0·50† | − 0·30 ± 0·58† | 33·8 ± 3·7† | 132·4 ± 11·7† | 13·0 ± 3·0† | 120·9 ± 14·2† |
| Zl-4 | 20 | 8 | 27·1 ± 0·99 | 31·5 ± 0·57 | + 4·40 ± 0·99 | 16·6 ± 3·2 | 259·1 ± 20·6 | 6·5 ± 1·3 | 142·6 ± 11·9 |
| Ratio Zl-4/Zs-4 | | | 0·98 | 1·15 | | 0·49 | 1·96 | 0·50 | 1·18 |

* See Table 1 for further details. † Standard Error.

*Groups Js-1 and Js-2.* (Groups *Junco* short-day 1 and *Junco* short-day 2. See Table 1.) These groups contained twenty birds in adult plumage maintained for 8 or 9 days on 8-hr photoperiods in small cages after transfer from outdoor aviaries on 30 November and 15 February, respectively. Groups Js-1 and Js-2 serve as short-day control groups for groups Jl-1, Jl-2 and Jl-3. The data are summarized in Tables 1 and 2 and in Fig. 3.

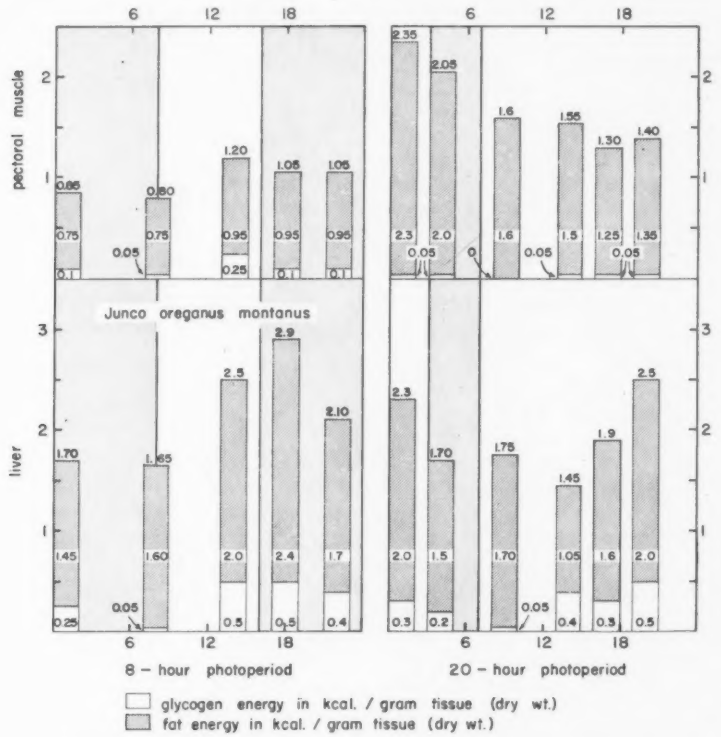
pectoral muscle
liver
Junco oreganus montanus
8 – hour photoperiod
20 – hour photoperiod
glycogen energy in kcal. / gram tissue (dry wt.)
fat energy in kcal. / gram tissue (dry wt.)

FIG. 2. *Junco oreganus montanus.* Energy reserves in pectoral muscle and liver in short and long daily photoperiods, mean values. No. of birds: 8 hr, 20; 20 hr, 34.

These data suggest that the metabolic adaptations for migration, as observed in *Zonotrichia leucophrys gambelii*, are rather poorly developed in the population of *Junco oreganus montanus* from which our experimental birds were obtained. The increase in weight following photoperiodic treatment is much smaller (Table 1) and the diurnal cycles in hepatic glycogen and fat appear to be relatively unaffected by the photoperiodic treatment. On the other hand the decrease in glycogen and increase in fat in the pectoral muscle in the 20-hr birds (Fig. 3) is similar to, although less conspicuous than, that of *Zonotrichia leucophrys gambelii.*

3. *Passer domesticus*

*Groups Pl-1 and Pl-2.* (Groups *Passer* long-day 1 and *Passer* long-day 2. See Table 1.) These groups contained twelve and twenty-five adults, respectively, which were subjected to 20-hr daily photoperiods for 21 days beginning on 23 January and 2 February (Table 1). On the twenty-second day the birds were killed at selected intervals as indicated in Fig. 4. Since there were no apparently significant differences, the data for these two groups have been combined in Fig. 3 and Table 3. The mean changes in body weights are recorded in Table 1.

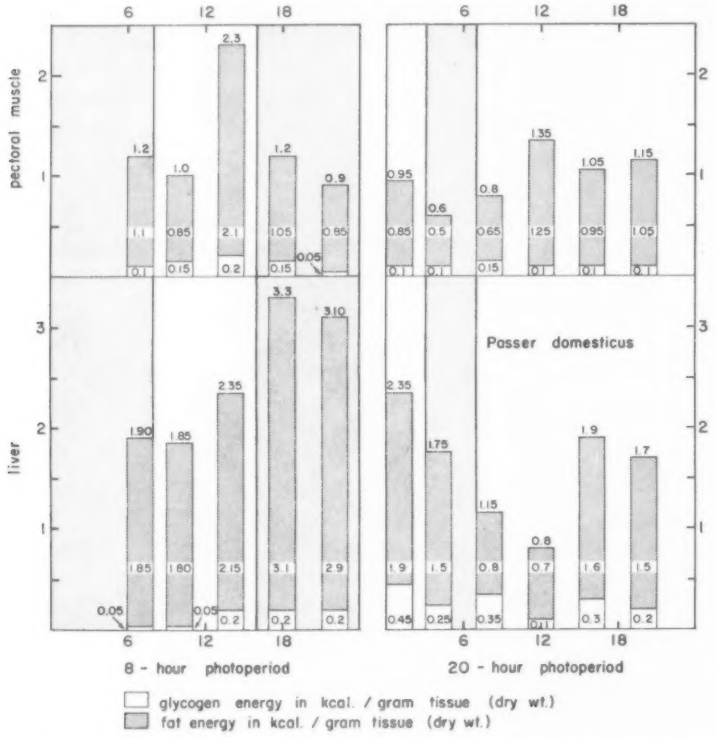


FIG. 3. *Passer domesticus.* Energy reserves in pectoral muscle and liver in short and long daily photoperiods, mean values. No. of birds: 8 hr, 24; 20 hr, 37.

*Groups Ps-1 and Ps-2.* (Groups *Passer* short-day 1 and *Passer* short-day 2. See Table 1.) These groups contained twelve adults each maintained for 7 or 8 days on 8-hr photoperiods beginning 30 November and 19 January, respectively. They serve as a short-day control for groups *Pl*-1 and *Pl*-2. The data are summarized in Tables 1 and 3 and in Fig. 4.

These data show that the photoperiodically induced metabolic changes which are clearly evident in *Zonotrichia leucophrys gambelii*, and somewhat less striking in

*Junco oreganus montanus*, are completely lacking in *Passer domesticus*. The only apparent effect of the long photoperiod in *Passer domesticus* appears to be a reduction in the amount of hepatic fat, an effect precisely opposite to the effect on *Zonotrichia leucophrys gambelii*.

TABLE 5—ESTIMATED ENERGY RESERVES AS FAT AND CARBOHYDRATE AT THE END OF THE DAY DURING MID-WINTER AND DURING THE MIGRATORY PERIOD.*
*Zonotrichia leucophrys gambelii*

| | Mid-winter (January–February) | Migration (Late April–early May) |
|---|---|---|
| Mean body wt. (g) | 27 | 32 |
| Mean ether-extractable fat (g) | 2·5 | 7·5 |
| Total energy reserve in fat (kcal) | 24 | 71 |
| Fat energy in skeletal muscle (kcal) | 0·07 | 1·0 |
| Fat energy in liver (kcal) | 0·2 | 0·5 |
| Energy reserve in glycogen (kcal) | | |
| Glycogen energy in skeletal muscle (kcal) | 0·02 | 0·02 |
| Glycogen energy in liver (kcal) | 0·07 | 0·03 |
| Energy reserve in blood glucose (kcal) | 0·04 | 0·03 |
| Total carbohydrate energy reserve (kcal) | ca. 0·15 | <0·1 |
| Total fat and carbohydrate energy reserve (kcal) | 24 | 71 |

* Estimated on the basis of data from Oakeson (1953), McGreal & Farner (1956), King (1957), King & Farner (1959) and Kamemoto, King & Farner (unpublished).

## IV. ANALYSES OF BIRDS TAKEN FROM WILD FLOCKS

These studies were restricted to *Zonotrichia leucophrys gambelii*. *Group Zww* (Group *Zonotrichia* wild winter) consisted of sixteen birds from wintering flocks in the Snake River Canyon in January and February 1958. *Group Zwp* (Group *Zonotrichia* wild premigratory) contained nine birds from a flock in the Snake River Canyon which had been under observation for several weeks. The birds were taken on 17 April, approximately 1 week before the flock left the area. *Group Zwm* (Group *Zonotrichia* wild migratory) was taken from a migratory flock in the vicinity of Pullman on 25 April 1958. This group consists of eight birds. All of the birds in these three groups were taken within 3 hr of sunset. The data are compiled in Table 2.

An inspection of these data reveals that between Group Zww and Group Zwm there is an enormous increase in energy stored in both pectoral muscle and liver, and furthermore, as in the cases of the experimental birds, the increase is the result of an increased deposition of fat. As in the case of the long-day experimental birds (Zl-1, Zl-2, Zl-3, Zl-4), glycogen is an insignificant component of the energy reserve in the liver and pectoral muscles in the migrating birds.

## V. THE HISTOCHEMICAL OBSERVATIONS

Histochemical studies were restricted to *Zonotrichia leucophrys gambelii*. Sections were prepared from twenty-three birds killed at different times of the day from three groups: (1) birds subjected to 20-hr photoperiods; (2) birds subjected to 8-hr photoperiods; and (3) birds held in outdoor aviaries under natural conditions of photoperiod and temperature until summer. The results of these studies are summarized in Table 6 with illustrations of typical sections in Figs. 4–7. The

TABLE 6—HISTOCHEMICAL DEMONSTRATION OF HEPATIC GLYCOGEN UNDER EXPERIMENTAL AND NATURAL CONDITIONS. *Zonotrichia leucophrys gambelii*

| No. | Sex | Photoperiod | | Time on photoperiod | | Time of killing | Period of light or darkness (at time of killing) | Body wt. (g) | | Glycogen index* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Duration (hr) | Time | Dates | No. of days | | | Initial | Final | |
| | | | | | | | hr min | | | |
| 818 | ♂ | 8 | 1000–1800 | 25 I–11 VI | | 1615 | L 6 15 | 29·1 | 29·3 | 3 Fig. 7 (b) |
| 819 | ♂ | 8 | 1000–1800 | 25 I–11 VI | | 1633 | L 6 33 | 27·8 | 25·2 | 4 |
| 842 | ♀ | 8 | 1000–1800 | 25 I–11 VI | | 1626 | L 6 26 | 24·1 | 25·3 | 4 Fig. 7 (a) |
| 828 | ♂ | 20 | 0400–2400 | 23 V–11 VI† | 20 | 1646 | L 12 46 | 25·4 | 31·9 | 1 Fig. 7 (c) |
| 802 | ♂ | 20 | 0400–2400 | 23 V–11 VI† | 20 | 1639 | L 12 39 | 26·9 | 34·0 | 0–1 Fig. 7(d) |
| 845 | ♂ | 8 | 0700–1500 | 25 I–20 VI | | 0830 | L 1 30 | 26·7 | 26·7 | 1 |
| 787 | ♂ | 20 | 0700–0300 | 15 VI–20 VI† | 5 | 0830 | L 1 30 | 26·3 | 25·8 | 0–1 |
| 826 | ♀ | 8 | 0700–1500 | 25 I–26 VI | | 0830 | L 1 30 | 26·6 | 24·5 | 1 |
| 777 | ♂ | 20 | 0700–0300 | 15 VI–26 VI† | 10 | 0830 | L 1 30 | 27·7 | 30·7 | 0 |
| 778 | ♂ | 8 | 0700–1500 | 25 I–1 VIII | | 1430 | L 7 30 | 28·2 | 22·6 | 4 Fig. 4 (a) |
| 781 | ♂ | 8 | 0700–1500 | 25 I–1 VIII | | 1630 | D 1 30 | 29·6 | 28·6 | 4 Fig. 4 (b) |
| 776 | ♂ | 20 | 0700–0300 | 20 VII–2 VIII† | 15 | 0230 | L 19 30 | 32·5 | 37·9 | 1–2 Fig. 5 (a) |
| 843 | ♂ | 20 | 0700–0300 | 20 VII–2 VIII† | 15 | 0430 | D 1 30 | 29·2 | 37·4 | 1 Fig. 5 (b) |
| 838 | ♂ | 8 | 0700–1500 | 25 I–2 VIII | | 0630 | D 15 30 | | 29·4 | 2 Fig. 4 (c) |
| 807 | ♂ | 20 | 0700–0300 | 20 VII–2 VIII† | 15 | 0630 | D 3 30 | 29·3 | 32·6 | 0 Fig. 5 (c) |
| 825 | ♂ | 8 | 0700–1500 | 25 I–2 VIII | | 1030 | L 3 30 | 24·7 | 22·6 | 3 Fig. 4 (d) |
| 789 | ♀ | 20 | 0700–0300 | 20 VII–2 VIII† | 15 | 1030 | L 3 30 | 24·5 | 28·8 | 2 Fig. 5 (d) |
| 942 | ♂ | natural ca. 16 | ca. 0405–1945 | – 1 VIII | — | 1200 | L ca. 8 | — | 30·8 | 2 Fig. 6 (a) |
| 945 | ♀ | natural ca. 16 | ca. 0405–1945 | – 1 VIII | — | 1800 | L ca. 14 | — | 27·2 | 2 Fig. 6 (b) |
| 902 | ♀ | natural ca. 16 | ca. 0405–1945 | – 1 VIII | — | 2400 | D ca. 4 | — | 24·7 | 0–1 Fig. 6 (c) |
| 944 | ♀ | natural ca. 16 | ca. 0405–1945 | – 2 VIII | — | 0125 | D ca. 5 | — | 25·7 | 0 Fig. 6 (d) |
| 904 | ♀ | natural ca. 16 | ca. 0405–1945 | – 2 VIII | — | 0600 | L ca. 2 | — | 25·5 | 2 Fig. 6 (e) |
| 941 | ♀ | natural ca. 16 | ca. 0345–2005 | –16 VII | — | 1000 | L ca. 6 | — | 27·8 | 2 Fig. 6 (f) |

* An arbitrary index based on density and extent of glycogen stained with periodic acid–Schiff reagent; 0 means none detectable and 4 indicates maximum observed.

† These birds were maintained on 8-hr daily photoperiods (1000–1800) from mid-January until the 20-hr daily photoperiods were begun on the dates indicated.

diurnal cycle in hepatic glycogen is clearly evident with a maximum concentration near the end of the daily photoperiod in all three groups. As indicated by the chemical analyses also, the cycle is most pronounced in the 8-hr birds (Fig. 4) in which hepatic storage of glycogen was the greatest. Fig. 7 compares the glycogen

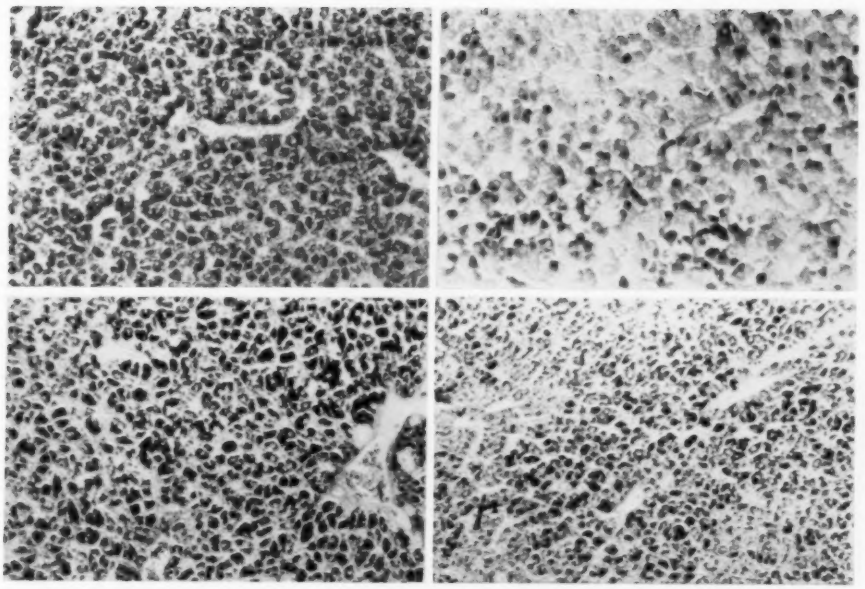


FIG. 4. *Zonotrichia leucophrys gambelii*. Histochemical demonstration of hepatic glycogen cycle in birds subjected to 8-hr daily photoperiods (0700–1500). (a) No. 778♂, 1430, 1 August 1957. Extensive glycogen deposition near end of daily photoperiod. (b) No. 781♂, 1630, 1 August 1957. Extensive glycogen 1½ hr after end of photoperiod. (c) No. 838♂, 0630, 2 August 1957. Reduced glycogen reserve near end of dark period. (d) No. 825♂, 1030, 2 August 1957. Increase in hepatic glycogen 3½ hr after beginning of photoperiod. Periodic acid—Schiff (×150).

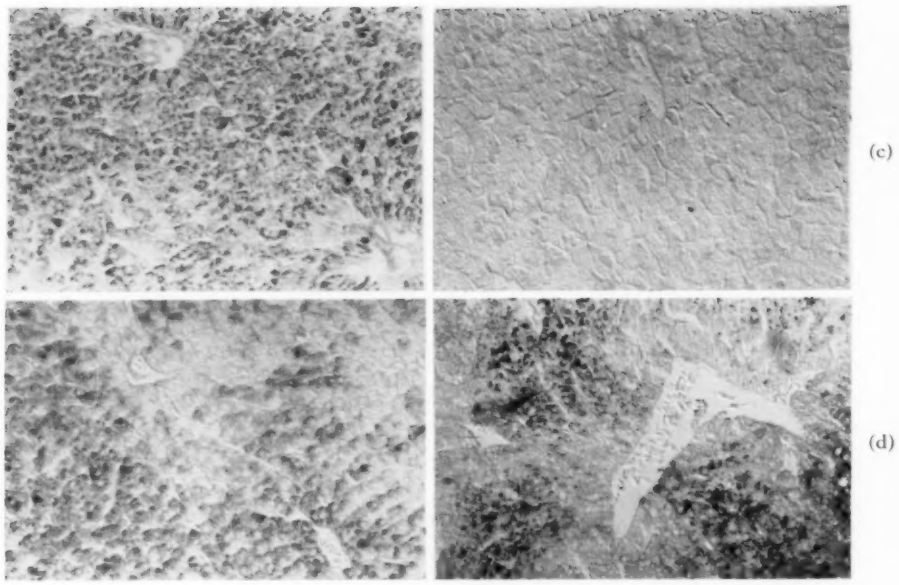


FIG. 5. *Zonotrichia leucophrys gambelii*. Histochemical demonstration of hepatic glycogen cycle in birds subjected to 20-hr daily photoperiods (0700–0300). (a) No. 776♂, 0230, 2 August 1957. Accumulation of glycogen near end of daily photoperiod. (b) No. 843♂, 0430, 2 August 1957. Glycogen reserve 1½ hr after end of photoperiod. (c) No. 807♂, 0630, 2 August 1957. Exhaustion of hepatic glycogen before end of dark period. (d) No. 789♀, 1030, 2 August 1957. Restricted reaccumulation of glycogen during photoperiod. Periodic acid–Schiff (×150).

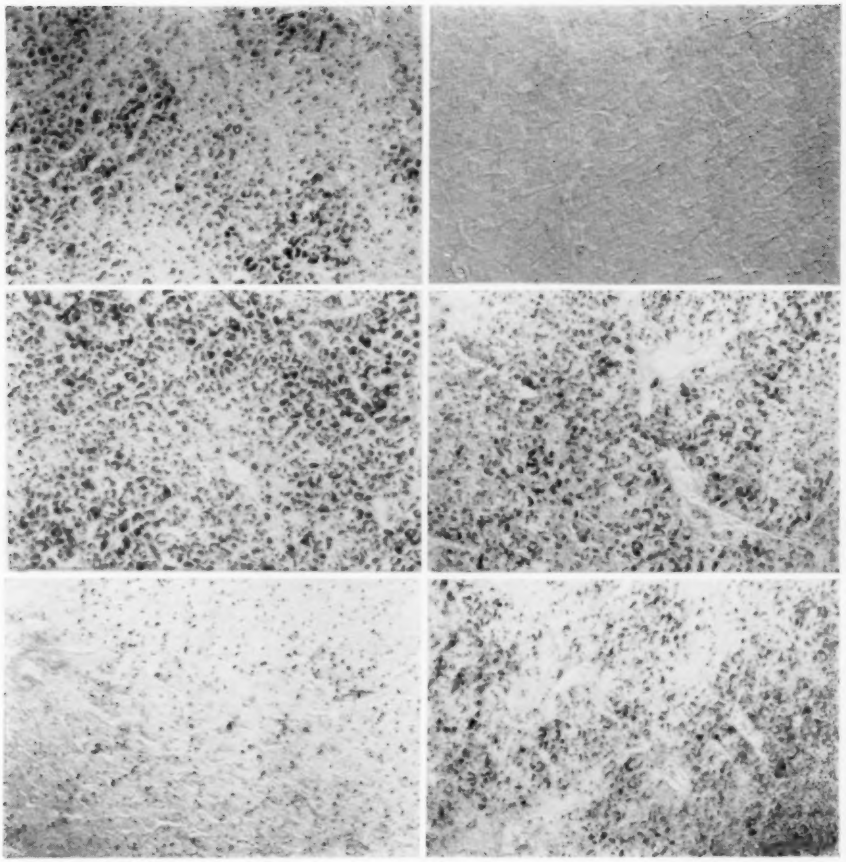


FIG. 6. *Zonotrichia leucophrys gambelii*. Histochemical demonstration of diurnal hepatic glycogen cycle in mid-summer in birds in large aviaries under natural conditions of temperature and photoperiod. (a) No. 942♂, 1200, 1 August 1957. (b) No. 945♀, 1800, 1 August 1957. (c) No. 902♀, 2400, 1 August 1957. (d) No. 944♀, 0125, 2 August 1957. (e) No. 904♀, 0600, 2 August 1957. (f) No. 941♀, 1000, 16 July 1957. Periodic acid–Schiff (×150).

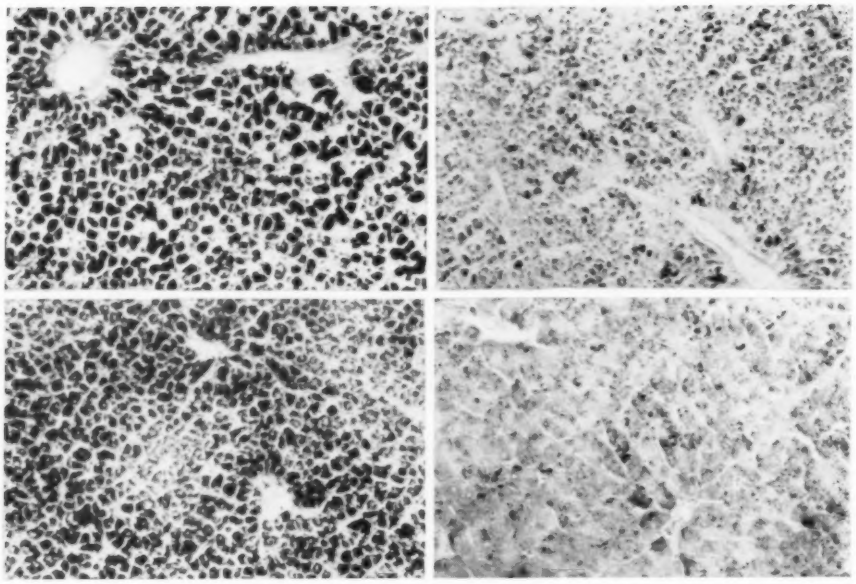


FIG. 7. *Zonotrichia leucophrys gambelii*. Histochemical comparison of glycogen storage in the livers of birds subjected to 8-hr and 20-hr daily photoperiods. (a) No. 842♂, 8-hr daily photoperiods (1000–1800), 1626, 11 June 1957. (b) No. 818♂, 8-hr daily photoperiods (1000–1800), 1615, 11 June 1957. (c) No. 828♂, 20-hr daily photoperiods (0400–2400) for 20 days, 1646, 11 June 1957. (d) No. 802♂, 20-hr daily photoperiods (0400–2400) for 20 days, 1639, 11 June 1957. Periodic acid–Schiff (×150).

accumulation during the photoperiod in short-day (Figs. 7 a, b) and long-day birds (Figs. 7 c, d). These may be compared with the chemical analyses as presented in Fig. 1, Table 2 and Table 3.

## VI. DISCUSSION

The data obtained from the experiments with *Zonotrichia leucophrys gambelii* on short days indicate that, with abundant food available *ad libitum*, there is an appreciable storage of glycogen in pectoral muscle and in liver and that there is a typical diurnal cycle of storage. In liver the cycle is apparently similar to that observed more precisely by Elfvin *et al.* (1955) in the domestic fowl. *However, when these birds are experimentally photo-stimulated into migratory state, there is a marked decrease in the glycogen content of pectoral muscle and liver and an even more striking increase in the fat content so that the total energy content of these tissues increases.* It is tempting to assume that this reduction in glycogen and increase in fat represents, in itself, a weight-saving scheme with respect to energy transport in migration, for it is obvious that energy transport in the form of fat could have an obvious advantage in carrying more calories per gramme. One can logically accept the thesis that the storage of fat in the subcutaneous and visceral reservoirs in a sense is an adaptation in this respect. Actually, however, the reduction in glycogen in the bird in migratory state is not important in this respect because of the insignificance of glycogen energy in the total energy reserve at all times. This becomes obvious from the calculations presented in Table 5. Therefore the reduction in glycogen in muscle and liver must be rationalized in relation to the accelerated lipogenesis. However, regardless of the nature of the interrelationships among fat and glycogen concentrations in the liver and pectoral muscles of photoperiodically stimulated birds, the net result is still the storage of a greater percentage of the energy as fat. That the change in quantity and distribution of energy in the pectoral muscles still represents a part of an adaptive scheme for migratory flight may be argued also from the relatively slight decrease in glycogen and slight increase in fat in the thigh muscles (Table 4). Further support for the hypothesis of a photoperiodically induced alteration in the pattern of energy storage for migration comes from the comparisons with *Junco oreganus montanus* and *Passer domesticus* (Table 2, Figs. 3 and 4). In the case of the former, in which the distance between wintering and breeding areas is relatively small, the photoperiodically induced fat storage, as indicated by change in body weight (Table 1), is much less extensive and the diurnal cycles in fat and glycogen content of the liver appear to be relatively unaffected. However, the increase in fat and decrease in glycogen in pectoral muscle is similar to that of *Zonotrichia leucophrys gambelii*, although smaller (Fig. 3, Table 2). It is finally important to emphasize that in the non-migratory *Passer domesticus* there is no photoperiodically induced alteration in glycogen storage and that the amount of fat stored in liver and pectoral muscle actually appears to be decreased by long daily photoperiods.

The low concentrations of glycogen in pectoral muscle and liver in *Zonotrichia leucophrys gambelii*, despite increased food intake (King & Farner, 1956), could be

the result of an increased rate of glycolysis which, in turn, perhaps could have significant acetate-sparing effect, thereby making more acetate available for fat synthesis. Superficially, at least, this resembles the observations that some types of tissue cultures are characterized by high rates of glycolysis and fat accumulation. (See, for example, Grossfeld, 1958.) It is also possible that the lower glycogen concentrations could be the result of an increased activity of the hexose monophosphate shunt. Sonka *et al.* (1959) have recently correlated increased shunt activity with human obesity and have suggested that shunt activity, because of its importance in the reduction of triphosphopyridine nucleotide, may be regarded as an indicator of the rate of lipogenesis. Also it has been demonstrated recently (Cohn & Joseph, 1959a) that force-fed rats accumulate more fat than rats with the same caloric intake *ad libitum*; this also appears to be correlated with a greater hexose monophosphate shunt activity (Cohn & Joseph, 1959b). It is important that the possibility of similar phenomena in *Zonotrichia leucophrys gambelii*, in this case as a result of a photoperiodically induced alteration in feeding pattern and increased caloric intake, be explored.



An examination of Table 2 reveals some important differences between the caged and wild birds. Explanations of these differences can only be speculative until further studies have been completed. Most striking is the very low level of glycogen in the pectoral muscles and liver of the wild winter birds (Group *Z*ww). Possibly this may be imposed by a less accessible food supply, by different temporal pattern of food intake, and/or by the necessity of spending greater quantities of readily mobilizable energy (glycogen) in the procurement of food than must be spent by the caged birds who need hop only a few centimeters to obtain rich food *ad libitum*. On the other hand it must be emphasized that in total energy reserves (principally fat) there is little difference between caged and wild birds in mid-winter (King & Farner, 1959) and similarly only slight differences in weight (Table 2). As noted in Table 5, the average wild bird in mid-winter has an energy reserve of about 24 kcal. According to the measurements of King (1957) it can be estimated that a *Zonotrichia leucophrys gambelii* requires about 8 kcal to maintain itself for 15 hr in an air temperature of 0°C. Thus it appears that there is a very adequate caloric reserve for mid-winter conditions. Possibly also the method of procurement of the samples of wild birds may have a bearing on the lower glycogen content of muscle and liver since our system for capturing them in mist nets involves driving them for distances of as much as a kilometer before they are caught in the nets. It is possible that this amount of exercise results in a depletion of glycogen in liver and muscle; but this must be the subject of further experiments.

It is to be noted further that the wild migratory birds (Group *Z*wm) have somewhat lower total energy reserves particularly in pectoral muscle. Although this must be the subject of further investigation, there is again the possibility of the greater availability of food and the almost complete lack of flight activity in the case of the long-day caged birds (Groups *Z*l-1, *Z*l-2, *Z*l-3, *Z*l-4). Again to be considered is the possible reduction in energy content of the pectoral muscles as a result of the activity involved in the precapture chase. It seems apparent that

the data are consistent with a hypothesis that one aspect of the change in metabolism which accompanies the photoperiodically induced vernal premigratory fat deposition involves a failure to accumulate appreciable quantities of glycogen in pectoral muscle and liver.

It is of further interest to observe that the pectoral muscles of all three species at all times contain considerable quantities of fat regardless of the nature of the photoperiod. This is consistent with the observations of George and his colleagues (George & Jyoti, 1955a, b; George & Naik, 1957a) who have demonstrated that the pectoral muscles of birds contain two types of fibers, a *narrow type* which contains large globules of fat and granules of lipoprotein and a *broad type* with sparse inclusions, glycogen, and only very minute fat globules. It has been shown further that the narrow type of fiber predominates in birds capable of sustained flapping flight and that with sustained activity the fat globules disappear. The narrow type is less frequent or absent from the pectoral muscles of birds incapable of sustained flapping flight; it is also lacking in leg muscles. One may cautiously propose the hypothesis that in these finches either glycogen or fat may be used directly by the muscles for fuel and that in *Zonotrichia leucophrys gambelii*, and possibly in *Junco oreganus montanus*, an important aspect then of the photoperiodically induced alteration in metabolism is a more extensive or even exclusive use of fat (or fatty acids) directly by the pectoral muscles. Histochemical studies of the pectoral muscles of these finches in migratory and non-migratory state and in comparison with *Passer domesticus* are obviously essential. The investigations of Gruber & Ruys (1951) and George & Jyoti (1957) show quite clearly that even in birds with considerable glycogen reserves sustained flight activity draws most of its energy from fat reserves. The same appears to be true in flight of at least one of the bats, *Hipposideros speoris* (George & Naik, 1957b; George & Jyoti, 1958). Among insects, it has been shown that sustained flight depends on the use of fat or fatty acids by the migratory locust, *Schistocerca gregaria* (Krogh & Weis-Fogh, 1951; Weis-Fogh, 1952) and probably also by the noctuid moth, *Prodenia eridania* (Zebe, 1958). This evidence from other species, together with the increased fat deposition and negligible glycogen reserves in *Zonotrichia leucophrys gambelii* (Table 5), can lead to no other conclusion than that the energy for migratory flight must come almost exclusively from fat. If the birds in Group Zwm may be regarded as typical of migratory birds at the end of the day preceding a night of migration, it means that the total glycogen-energy in pectoral muscle and liver (calculated as above) available at the beginning of a migratory flight is about 0·05 kcal. According to the data of King (1957) this would provide energy for resting metabolism for no more than about 5 min at 20°C. Thus it is clear that almost all of energy for flight must come from fat. The fact that fat is stored in extensive quantities in pectoral muscle and indeed within the fibers themselves (George & Naik, 1957a) argues that fat, or fatty acids (George & Scaria, 1958), are used directly as sources of energy, although this remains to be confirmed by more direct experiments. However, it should be observed that direct utilization of fatty acids by mammalian cardiac muscle (e.g. Bing, 1956; Ungar *et al.*, 1955;

Gordon, 1957; Gordon & Cherkes, 1956) and by mammalian skeletal muscle (e.g. Weinhouse *et al.*, 1950; Wertheimer & Ben-Tor, 1952; Andres *et al.*, 1956; Gordon, 1957; Hansen & Rutter, 1952) appears now to be well established.

The relatively high ratio of fat energy to glycogen in the livers of all three species is consistent with the observations of Fisher & Bartlett (1957) on two other passerine species, *Agelaius phoeniceus* and *Sturnus vulgaris*.

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# INTERMEDIARY CARBOHYDRATE METABOLISM OF *TRIATOMA INFESTANS* (INSECTA; HEMIPTERA)*—I. GLYCOLYTIC AND PENTOSE PHOSPHATE PATHWAY ENZYMES AND THE EFFECT OF DDT†

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**Abstract**—(1) Cell-free preparations obtained from thoraces and legs of *T. infestans* glycolyse glucose, as well as HDP, with the production of pyruvate and α-glycerophosphate in equimolecular amounts in the proportion of 1 mole of pyruvate and 1 mole of α-glycerophosphate per mole of glucose utilized. Added HDP is necessary for optimal activity, but the effect of its omission can be partially reversed by increasing the concentration of added ATP. Fluoride is not required for glucose phosphorylation.

(2) Cell-free extracts contain enzymes for glycolysis. GAP dehydrogenase is markedly sensitive to iodoacetate and shows appreciable activity in the absence of added L-cysteine. Lactic dehydrogenase activity is negligible, which explains the non-accumulation of lactate as an end-product of glycolysis.

(3) The presence of the pentose phosphate pathway in *T. infestans* is indicated by the presence of several reactions of this pathway in cell-free extracts; 6-PG dehydrogenase and probably G-6-P dehydrogenase appear to be rate-limiting enzymes for carbohydrate degradation through the pentose pathway.

(4) The enzyme content of cell-free extracts is related to the developmental stage of the insect and also to the sex.

(5) *In vivo* topically applied DDT (300 μg per specimen) inhibits the glycolytic pyruvate production by cell-free preparations of male and nymph specimens after 22 hr of exposure. Pyruvate production is restored to normal after 48 hr of exposure, but probably some degree of inhibition of α-glycerophosphate production persists in male specimens. This effect is shared by DDE, a non-toxic DDT-analog.

(6) Hexokinase, G-6-P dehydrogenase, 6-PG dehydrogenase, aldolase, GAP dehydrogenase, Gly-P dehydrogenase, phosphopyruvic kinase and ribolytic activity are inhibited by DDT in male specimens after 48 hr of exposure to DDT. Phosphopentose isomerase apparently is not inhibited. On the other hand, only G-6-P dehydrogenase and aldolase are inhibited in nymph specimens by DDT.

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† Abbreviations: DDT, 2, 2-bis-(p-chlorophenyl)-1, 1, 1-trichloroethane; DDE, 2, 2-bis-(p-chlorophenyl)-1, 1-dichloroethylene; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate; F-6-P, fructose-6-phosphate; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; GAP, glyceraldehyde-3-phosphate; Gly-P, alpha-glycerophosphate; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5-phosphate; $DPN^+$, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; $TPN^+$, oxidized triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; HDP, fructose-1, 6-diphosphate; DAP, dihydroxyacetone phosphate; 2-PG, 2-phosphoglycerate; FAD, flavinadenine dinucleotide; "Tris" buffer, tris (hydroxymethyl) amino methane; TCA, trichloroacetic acid; PCA, perchloric acid; DPI, 2, 6-dichlorophenol indophenol; IAA, iodoacetate; Pi, inorganic phosphorus.

## INTRODUCTION

STUDIES on the fundamental biochemical processes in insects are not only a contribution to general and comparative biochemistry, but also may constitute a basis for a better understanding of the mode of action of insecticides and the mechanisms of resistance of insects towards insecticides. The view that insecticides kill by interfering with key metabolic reactions of insects is gaining wide support (Kearns, 1956). Furthermore, the mechanism(s) of resistance are also very likely primarily biochemical.

Although a relatively clear pattern of the intermediary carbohydrate metabolism of vertebrates and microorganisms was already established several years ago, our knowledge regarding the enzymatic reactions leading to synthesis and degradation of carbohydrates in insects is far from satisfactory. Conclusive evidence for the operation of the Embden–Meyerhof scheme in housefly has been obtained only recently (Chefurka, 1954). The presence of reactions of the pentose phosphate pathway has been established so far in the housefly (Chefurka, 1955, 1957) and the pea aphid *Macrosiphum pisi* (Newburgh & Cheldelin, 1955). As far as the reactions of the Krebs cycle are concerned a similar sequence to that established in higher animals has been shown to occur in insects (Winteringham & Harrison, 1956; Silva *et al.*, 1958). Oxidative phosphorylation was first demonstrated in housefly sarcosomes by Sacktor (1954). However, it must be emphasized that, in spite of fragmentary data suggesting the presence of the above biochemical mechanisms outlined in several other insects, conclusive evidence of the presence of such pathways exists essentially only for the housefly and the American cockroach (Rockstein, 1957).

Since no data are available on the intermediary metabolism of *Triatoma infestans*, the present studies were undertaken as a basis for further work at the biochemical level on the mode of action of DDT in this insect as well as with the aim of elucidating the marked differences in susceptibility towards DDT exhibited by the various developmental stages of *T. infestans*. The experiments to be reported here yield evidence for the operation of phosphorylative glycolysis as a mechanism of glucose degradation by cell-free preparations of *T. infestans*, pyruvate and Gly-P being the major end-products of glycolysis. Furthermore, the presence of reactions of the pentose phosphate pathway has been substantiated. Whenever possible, comparisons of *T. infestans* enzymes in various developmental stages as well as with other insect enzymes have been made. Experiments on the effect of DDT and DDE on the glycolytic and pentose phosphate enzymes of *T. infestans*, although unsuccessful as to pinpoint DDT action, will be also reported.

## METHODS

### *Insect material*

Laboratory reared *T. infestans* specimens were used throughout. They were maintained at 27°C and a relative humidity of 50 per cent in 50 ml beakers containing a piece of folded filter paper at the bottom. Most of the experimental work was done with adults and third instar larvae (nymphs), although first instar

larvae and 1-week-old eggs were also occasionally used. Insecticides dissolved in acetone were topically applied to the ventral abdominal region of the insects by means of a micropipette. Control groups were treated only with acetone. After the application of the insecticide, the insects were kept as described above during variable periods of time as indicated in Tables. The insects were starved at least 1 week before being used.

*Enzyme preparation*

The thoraces and legs of either pre-chilled larval, nymph or adult *T. infestans* were homogenized in 0·15 M KCl in a mortar with the aid of ballotine at 2°C and extracted during 60 min at the same temperature. The resultant brei (Fraction I) was filtered through cheesecloth to remove pieces of cuticle and then centrifuged at 20,000 *g* for 60 min at 2°C. The supernatant fluid thus obtained, filtered through Whatman No. 1 filter paper and diluted with 0·15 M KCl to an appropriate volume, was used as a source of enzyme for most of the work (Fraction II). When insects previously treated with DDT or DDE were used, prior to killing them for obtaining the thoraces and legs, the insects were rinsed three times individually in about 20 ml of fresh acetone to eliminate any external DDT or DDE. Whole egg extracts were prepared in a similar manner.

*Analytical procedures*

Overall glycolysis was estimated as described elsewhere (Agosin & Aravena, 1959a, b). A glycolytic reaction mixture patterned after Le Page (1948) was used. Pyruvic (Friedemann & Haugen, 1943) and lactic (Barker & Summerson, 1941) acids were determined in aliquots of the reaction mixture after deproteinization with cold TCA. The net uptake of glucose was estimated in the neutralized TCA supernatant fluid with glucose oxidase (Saifer & Gerstenfeld, 1958). Inorganic phosphate was determined according to Le Page (1948b) and Gly-P as described by Leva & Rapoport (1943). The 2,4-dinitrophenylhydrazone derivative of pyruvic acid was identified by paper chromatography with *t*-amyl alcohol, ethyl alcohol, and water as solvent (Altmann *et al.*, 1951). Pyruvic acid was also enzymatically identified by its reduction in the presence of DPNH and rabbit muscle lactic dehydrogenase. Volatile fatty acids were estimated quantitatively by steam-distillation and titration (Bueding, 1949), acetyl phosphate by the hydroxamate method of Lipmann & Tuttle (1945). Ethyl alcohol was measured according to Henry *et al.* (1948); fructose by the method of Roe (1934); ketopentoses, ketohexoses and trioses by the procedure of Dische & Borenfreund (1951) as modified by Axelrod & Jang (1954). Trioses were also estimated as alkali-hydrolysable phosphorus (Axelrod *et al.*, 1952). Ribose was determined according to Mejbaum's procedure (1939). Glycerol was estimated according to Burton (1957). Its formation as an end-product of glycolysis was also investigated by paper chromatography, with isopropanol, pyridine, acetic acid and water (8 : 8 : 1 : 4 v/v) as solvent. Glucokinase activity was measured as described elsewhere (Agosin & Aravena, 1959a) or by determining the glucose utilization with glucose oxidase



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(Saifer & Gerstenfeld, 1958) from the difference between the free glucose concentration of control and final samples. G-6-P and 6-PG dehydrogenases were assayed essentially according to Seegmiller & Horecker (1951); R-5-P isomerization to Ru-5-P by the procedure of Axelrod & Jang (1954); ribokinase by the method of Horecker *et al.* (1954). Phosphopyruvic kinase was estimated following the formation of pyruvate in the presence of ADP and $Mg^{2+}$, with 2-PG as substrate. Details concerning other enzymatic assay methods have been previously reported (Agosin & Aravena, 1959a, b). Rabbit muscle lactic dehydrogenase was prepared according to Kornberg & Pricer (1951). R-5-P, prepared by the method of Le Page & Umbreit (1943) or a commercial preparation, were used. D-Ru-5-P was prepared by the enzymatic oxidation of 6-PG (Horecker & Smyrniotis, 1951). The other reagents were obtained from commercial sources. Unless otherwise stated, potassium salts prepared by conventional methods were used. All substrates and coenzymes were assayed for purity and corrected values were used in reporting concentrations. All spectrophotometric determinations were done in a Beckman Model DU spectrophotometer. Nitrogen was determined by a micro-Kjeldahl procedure.

## RESULTS

### *Overall glycolysis*

When Fraction II derived from male, female or nymph specimens was allowed to glycolyse in the presence of a fortified medium, significant amounts of pyruvic acid were produced, approximately 1 mole per mole of glucose utilized (Table 1).

TABLE 1—GLYCOLYSIS IN VARIOUS DEVELOPMENTAL STAGES OF *Triatoma infestans*

| Stage | − Δ-glucose | + Δ-pyruvate μmoles/mg N /hr | + Δ-lactate | + Δ α-glycero-phosphate |
|---|---|---|---|---|
| Adult males | 2·43 ± 0·32 | 2·65 ± 0·33 | 0·10 ± 0·03 | 2·63 ± 0·30 |
| Adult females | 0·92 ± 0·10 | 0·94 ± 0·14 | 0·12 ± 0·05 | ——— |
| Nymphs (third instar larvae) | 1·59 ± 0·46 | 1·88 ± 0·54 | 0·06 ± 0·05 | 2·37 ± 0·33 |

The main compartment of the warburg flasks contained: Glucose, 10·0 μmoles; HDP, 2·0 μmoles; $DPN^+$, 2·4 μmoles; $MgCl_2$, 5·0 μmoles; Nicotinamide, 15·0 μmoles; $KHCO_3$, 50·0 μmoles; 1·0 ml of Fraction II (0·8–1·2 mgN). The sidearm contained: $KH_2PO_4$ buffer, pH 7·4, 10·0 μmoles; ATP, 8·0 μmoles. Incubated at 37° during 60 min. Atmosphere, 95% $N_2$–5% $CO_2$. Reaction stopped with 1·0 ml 10% TCA. Final volume, 2·0 ml. The figures after the ± sign correspond to the standard error of the mean.

The identity of pyruvic acid as the keto acid produced during glycolysis was established by comparing the chromatographic behaviour of the 2,4-dinitrophenylhydrazones of the reaction product and of an authentic sample of pyruvic acid. Furthermore, its formation was enzymatically substantiated by its reduction by DPNH in the presence of rabbit muscle lactic dehydrogenase (Fig. 1). On the other hand, lactic acid was produced in only very small amounts (Table 1).

Apparently, the limiting enzyme for the formation of lactate is lactic dehydrogenase, reoxidation of DPNH by pyruvate or reduction of $DPN^+$ by lactate catalysed by either Fraction I or II being negligible. Since the reductive step represented by lactic dehydrogenase appeared to be of minor importance in glycolysis, the possibility that other end-products in addition to pyruvate were produced during glycolysis was investigated. The reaction catalysed by alcohol dehydrogenase was ruled out as the reductive step since Fraction I or II showed only traces of alcohol dehydrogenase activity, either by the reduction of $DPN^+$ or $TPN^+$ in the presence of acetaldehyde. Furthermore, no measurable production of ethyl alcohol was

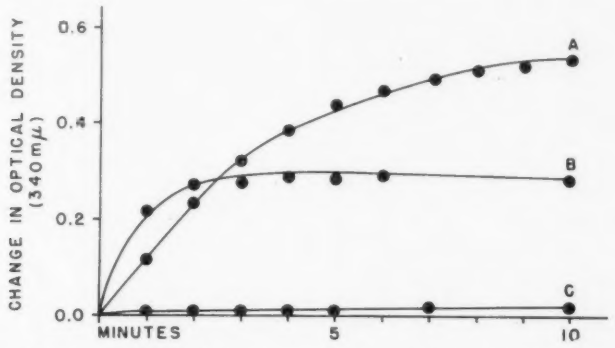


FIG. 1. Reoxidation of DPNH by pyruvate produced during anaerobic glycolysis by *Triatoma infestans* cell-free extracts in the presence of rabbit muscle lactic dehydrogenase. Initial and final reaction mixtures under the conditions of Table 1 were deproteinized with TCA, the supernatant fluid neutralized with NaOH and aliquots taken for the enzymatic determination of pyruvate.

Curve A: 100·0 μmoles $KH_2PO_4$ buffer, pH 7·0; 0·2 μmoles DPNH; 1·5 units lactic dehydrogenase; 0·2 ml neutralized supernatant fluid after incubation; curve B: same as A but with 0·1 ml supernatant fluid; curve C: same as A but with 0·2 ml supernatant fluid before incubation. The blank cuvette contained $H_2O$; final volume, 3·0 ml. The *Triatoma infestans* extracts (Fraction II) were derived from male specimens.

$$DPNH + H^+ + \text{pyruvate} \rightleftharpoons DPN^+ + \text{lactate}.$$

observed under the conditions of Table 1. The well-known Neuberg's type II fermentation, where glycerol as well as acetaldehyde are formed by fermenting yeast extracts in the presence of sulfite, suggested the possibility that glycerol could also be produced under conditions of Table 1. However, neither by chemical nor by chromatographic procedures (see under "Methods") was glycerol production detected. Furthermore, in a system containing in a final volume of 3·0 ml 100 μmoles of glycerol, 2·4 μmoles of $DPN^+$, and 33 μmoles of pyrophosphate buffer, pH 8·5, no reduction of $DPN^+$ or $TPN^+$ was observed by either Fraction I or II. Gly-P and pyruvate are the principal end-products of glycolysis in insect flight muscle (Chefurka, 1958; Gregg *et al.*, 1959). When Gly-P was determined according to Leva & Rapoport (1943), significant amounts were found (Table 1). Gly-P accumulated in the ratio of approximately 1 mole per mole of pyruvate

formed indicating that the reaction DAP + DPNH + $H^+$ → Gly-P + $DPN^+$ represents the reductive step in glycolysis of *T. infestans* thorax and leg extracts. Table 1 shows that the average rate of glycolysis is somewhat lower in nymph than male preparations and definitely lower in female extracts. It should be pointed out that under the experimental conditions of Table 1, no production of volatile fatty acids nor formation of acetyl phosphate was detected.

Comparative glycolytic rates of male and female extracts, under various experimental conditions, are shown in Table 2. Added HDP was necessary not

TABLE 2—COMPARISON OF GLYCOLYTIC ACTIVITY OF CELL-FREE EXTRACTS OF MALE AND FEMALE SPECIMENS OF *Triatoma infestans* UNDER VARIOUS EXPERIMENTAL CONDITIONS

| System | Males | | Females | |
|---|---|---|---|---|
| | − Δ-glucose | + Δ-pyruvate μmoles/mg N /hr | − Δ-glucose | + Δ-pyruvate |
| Complete | 2·44 ± 0·33 | 2·67 ± 0·36 | 0·92 ± 0·10 | 0·94 ± 0·14 |
| Minus HDP-glucose | | 0·0 | | 0·0 |
| Minus HDP | 1·27 ± 0·50 | 0·38 ± 0·07 | 0·27 ± 0·18 | 0·16 ± 0·07 |
| Minus HDP plus 20 μmoles ATP | 0·71 ± 0·20 | 0·48 ± 0·10 | 1·57 ± 0·39 | 0·46 ± 0·14 |
| Minus $DPN^+$ | 0·83 ± 0·37 | 1·02 ± 0·18 | 0·62 ± 0·38 | 0·31 ± 0·14 |
| Minus $Mg^{2+}$ | 0·93 ± 0·35 | 0·49 ± 0·01 | 0·35 ± 0·16 | 0·28 ± 0·06 |
| Plus IAA | 0·61 ± 0·40 | 0·46 ± 0·06 | 0·36 ± 0·02 | 0·33 ± 0·02 |

System as in Table 1. IAA at the final molarity of $1 \times 10^{+3}$ M as indicated.

only for optimal pyruvate production, but also for maximal glucose phosphorylation, especially in female extracts. No pyruvate was produced in the absence of both HDP and glucose. Apparently, pyruvate is partially produced from glucose and partially from HDP, as indicated by the data of Table 2. The effect of the omission of HDP was partially reversed by increasing the concentration of ATP from $4 \times 10^{-3}$ M to $1 \times 10^{-2}$ M. The latter effect was especially marked in female extracts (Table 2). Added fluoride was not required for glycolysis. In fact, glucose phosphorylation was practically identical in the presence or absence of $5 \times 10^{-2}$ M fluoride. Glycolysis was markedly inhibited by IAA at the final concentration of $1 \times 10^{-3}$ M (Table 2). This observation suggested that, contrary to what has been reported for other insect tissues (Chefurka, 1954), GAP dehydrogenase could be sensitive to this inhibitor, as shown later.

The effect of increasing the ATP concentration in the absence of HDP (Table 2) as well as the requirement of added HDP for optimal activity, suggested that the dephosphorylation of ATP may compete with the phosphorylation of glucose, a reaction catalysed by hexokinase. Fraction II derived from various developmental stages of *T. infestans* was shown to possess ATP-ase activity (Table 3). The ATP-ase activity was quite small in egg extracts, high in larvae and nymphs, and

intermediate in male and female extracts. ATP-ase activity was lower in females than in males, indicating sexual differences in addition to ontogenetic ones. ATP-ase activity in all cases was $Mg^{2+}$-dependent and fluoride-sensitive. It should be mentioned that Fraction I also had ATP-ase activity. The latter would probably correspond to a mixture of the sarcosome-bound ATP-ase described by Sacktor (1953) and the soluble ATP-ase of Fraction II, since fluoride was much less

TABLE 3—ATP-ASE ACTIVITY OF CELL-FREE EXTRACTS OF *Triatoma infestans**

| System | 1-week-old eggs | First instar larvae | Third instar larvae (nymphs) | Adult males | Adult females |
|---|---|---|---|---|---|
| Complete | 0·048 | 5·94 | 5·13 | 2·16 | 1·27 |
| Minus $Mg^{2+}$ | 0·0014 | 3·98 | 4·46 | 1·11 | 0·79 |
| Plus $F^-$ | 0·0027 | 3·06 | 2·86 | 1·76 | 0·39 |

* Measured in μmoles Pi per mg N.

Complete system: ATP, 1·0 μmole; Glycyl-glycine buffer, pH 7·5, 10·0 μmoles; $MgCl_2$, 5·0 μmoles; KF, as indicated, 5·0 μmoles; Fraction II 0·5–0·7 mg N. Final volume, 1·0 ml. Incubated at 37° for 30 min. Reaction stopped with 1·0 ml 10% TCA.

effective in inhibiting the ATP-ase activity of Fraction I than II, under similar conditions. Whether dephosphorylation of ATP by Fraction II is mediated through "true" ATP-ases or pyrophosphatases remains to be established.

*Enzymes of the glycolytic pathway*

Cell-free extracts (Fraction II) were shown to possess enzymes for glycolysis. Egg and larval extracts had rather low activity; therefore these studies were conducted with nymphs and adults of *T. infestans*. Evidence for the presence of hexokinase, adenylate kinase and G-6-P dehydrogenase was found on addition of ADP to the extracts, in the presence of $Mg^{2+}$ and $TPN^+$. When glucose was added, $TPN^+$ was reduced, a demonstration that ADP was being converted to ATP and AMP, the former then transferring its terminal phosphate group to glucose by the action of hexokinase; the G-6-P thus formed being in turn oxidized by $TPN^+$ by the action of G-6-P dehydrogenase. $DPN^+$ did not act as oxidizing agent in this system, since G-6-P dehydrogenase was $TPN^+$ dependent (Fig. 2), and no transhydrogenase activity was present in the extracts. Direct glucose oxidation was not observed when whole homogenates or Fraction II of nymph and adult stages were incubated in the presence of the substrate and $DPN^+$ or $TPN^+$ as possible oxidizing agents, nor was glucose oxidized in a system containing in a final volume of 3·0 ml, 0·25 mg FAD; 10·0 μmoles $MgCl_2$; 50·0 μmoles glycyl-glycine buffer, pH 7·1; 20·0 μmoles glucose; and 0·5 ml of 0·01% DPI. Under these conditions, no reduction of DPI was observed at 600 mμ. On addition of HDP to the extracts in the presence of arsenate, cysteine, and $DPN^+$, the latter was reduced, indicating the splitting of HDP by aldolase into GAP and DAP and the oxidation of GAP to arsenophosphoglycerate by GAP dehydrogenase.

A $DPN^+$-dependent Gly-P dehydrogenase was also present in Fraction II, $DPN^+$ being promptly reduced in the presence of Gly-P. The coupling of the latter enzyme to aldolase is shown in Fig. 3. When Fraction II was incubated in the presence of DPNH, the latter was reoxidized upon addition of HDP, further confirming the presence of aldolase and Gly-P dehydrogenase in the extracts.

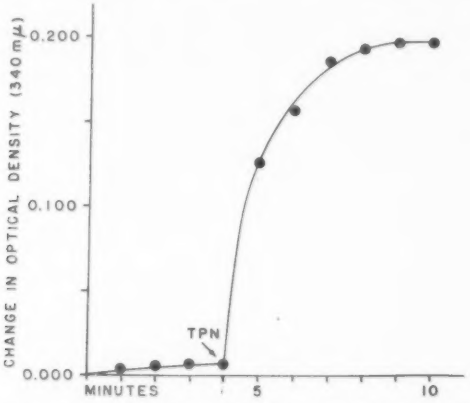


FIG. 2. Coenzyme specificity of *Triatoma infestans* G-6-P dehydrogenase. System: 20·0 μmoles glycyl-glycine buffer, pH 7·5; 10·0 μmoles $MgCl_2$; 1·2 μmoles $DPN^+$; 4·0 μmoles G-6-P to start the reaction; 350 μg N Fraction II derived from male specimens. The blank cuvette contained the same components with the exception of G-6-P. At the indicated time, 0·14 μmoles $TPN^+$. Final volume, 3·0 ml.

$$G\text{-}6\text{-}P + TPN^+ \rightarrow 6\text{-}PG + TPNH + H^+$$

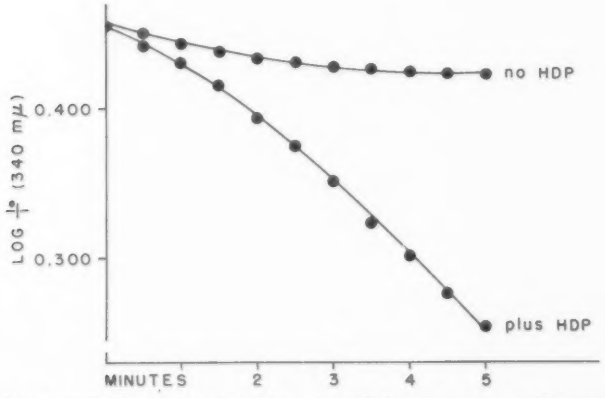


FIG. 3. Aldolase and Gly-P dehydrogenase in cell-free extracts of *Triatoma infestans*. System: 2·0 μmoles HDP to start the reaction; 0·2 μmoles DPNH; 100·0 μmoles $KH_2PO_4$ buffer, pH 7·4; 633 μg N Fraction II derived from female specimens. The blank cuvette contained $H_2O$. Final volume, 3·0 ml.

(1) $HDP \rightarrow DAP + GAP$

(2) $DAP + DPNH + H^+ \rightarrow Gly\text{-}P + DPN^+$

Sum: $HDP + DPNH + H^+ \rightarrow Gly\text{-}P + DPN^+$

The effect of IAA on glycolysis (Table 2) suggested that GAP dehydrogenase was sensitive to IAA. The data of Table 4 indicate that *T. infestans* GAP dehydrogenase is markedly sensitive to IAA. Concentrations lower than those reported by Chefurka (1954) as inactive on housefly GAP dehydrogenase, such as $6{\cdot}6 \times 10^{-5}$ M,

TABLE 4—THE EFFECT OF IAA ON GAP DEHYDROGENASE OF *Triatoma infestans*

| Additions | Final molarity | Change in optical density per min | Inhibition (%) |
|---|---|---|---|
| None | | 0·119 | |
| IAA | $1 \times 10^{-3}$ | 0·003 | 98 |
| IAA | $3{\cdot}3 \times 10^{-4}$ | 0·005 | 96 |
| IAA | $6{\cdot}6 \times 10^{-5}$ | 0·054 | 55 |

System as the complete system of Fig. 4, with 198 μg N. Extract (Fraction II) derived from male specimens.

produced 55 per cent inhibition of the *T. infestans* enzyme. Another difference from the housefly enzyme (Chefurka, 1954) is the practically absolute requirement of added —SH groups for activity of the housefly enzyme, whereas the *T. infestans* GAP dehydrogenase, although requiring added L-cysteine for maximal activity, shows an appreciable rate of $DPN^+$ reduction in the absence of L-cysteine (Fig. 4).

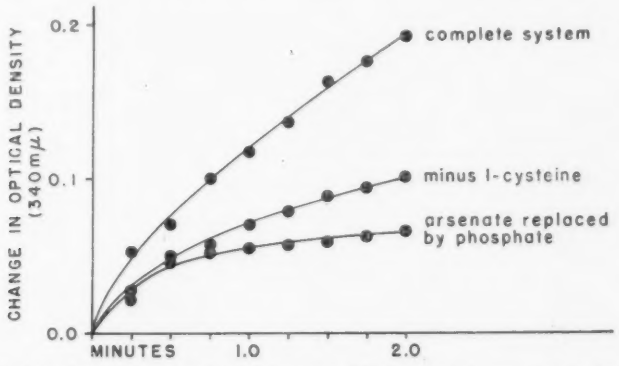


FIG. 4. GAP dehydrogenase in cell-free extracts of *Triatoma infestans*. Complete system: 0·5 μmoles GAP to start the reaction; 2·4 μmoles $DPN^+$; 10·0 μmoles arsenate; 10·0 μmoles L-cysteine; 100·0 μmoles pyrophosphate buffer, pH 8·5; 10·0 μmoles $KH_2PO_4$ buffer, pH 7·0 to replace arsenate as indicated; 172 μg N Fraction II derived from female specimens. Final volume, 3·0 ml. The blank cuvette contained the same components but GAP.

As expected, GAP dehydrogenase activity was also dependent on the presence of arsenate in the reaction mixture (Fig. 4). Fraction II had a high phosphopyruvic kinase activity, pyruvate being readily formed in a system containing in a final volume of 1·1 ml, 2 μmoles 2-PG, 5 μmoles $MgCl_2$, 8 μmoles ADP, and 20 μmoles "Tris" buffer, pH 7·1 (Table 6).

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*Enzymes of the pentose phosphate pathway*

6-PG oxidation was readily carried out by Fraction II in the presence of $TPN^+$; $DPN^+$ was not active in this system (Fig. 5). Optimum pH for 6-PG oxidation was around 8·5 with "Tris" or glycyl-glycine buffer, while optimum pH for G-6-P oxidation was around 7·5 with glycyl-glycine buffer. The Ru-5-P formed by the oxidative decarboxylation of 6-PG is known to be partially isomerized to R-5-P in different vertebrate (Horecker *et al.*, 1954), invertebrate (unpublished

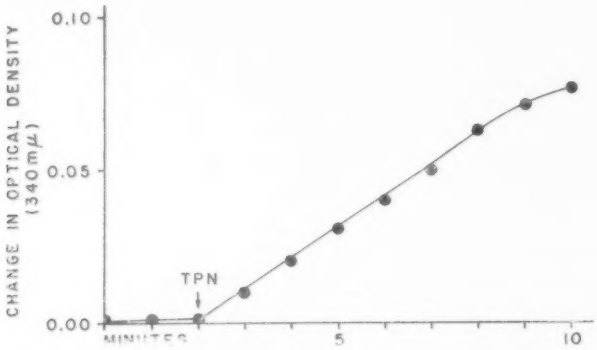


Fig. 5. 6-PG dehydrogenase in *Triatoma infestans* cell-free extracts. System: 100·0 μmoles "Tris" buffer, pH 8·5; 10·0 μmoles $MgCl_2$; 0·14 μmoles $TPN^+$ as indicated; 1·2 μmoles 6-PG to start the reaction; 576 μg N Fraction II derived from male specimens; final volume, 3·0 ml. Blank cuvette with same components with the exception of 6-PG.

$$6\text{-PG} + TPN^+ \rightarrow \text{Ru-5-P} + CO_2 + TPNH + H^+.$$

data of the authors) and plant (Axelrod & Jang, 1954) tissues. The enzyme catalysing this isomerization is also present in *T. infestans* extracts. Upon incubation of Fraction II in the presence of R-5-P, the optical density in the cysteine-carbazole test (Dische & Borenfreund, 1951) at 545 mμ increased with time, indicating the formation of ketose from R-5-P. Concomitantly, R-5-P disappearance could be demonstrated by the orcinol test (Mejbaum, 1939). Indication that R-5-P could be further metabolized, probably via transketolase-transaldolase, was obtained upon incubation of cell-free extracts with R-5-P at room temperature; when after 30 min $TPN^+$ was added, there was reduction of $TPN^+$, indicating the conversion of R-5-P to G-6-P. Further evidence of the conversion of R-5-P to hexose was obtained by estimating the formation of fructose (Roe, 1934) in aliquots of the reaction mixture under the same conditions at timed intervals. Fructose was rapidly formed, indicating that F-6-P is isomerized to G-6-P which is then oxidized by $TPN^+$ by the action of G-6-P dehydrogenase. Under these conditions, Dische's test (Dische & Borenfreund, 1951) gave indications of the formation of trioses, in addition to ketopentoses and ketohexoses. Ribokinase activity could be demonstrated in Fraction I or II of *T. infestans*. Free pentose rapidly disappeared when the extracts were incubated with ribose, ATP, $Mg^{2+}$ and L-cysteine, indicating the phosphorylation of ribose to R-5-P.

*The effect of DDT and DDE*

Male and nymph *T. infestans* specimens markedly differ in their susceptibility towards DDT, males being more susceptible than nymphs, as shown in Table 5. It should be added that DDE was apparently non-toxic to either male or nymph

TABLE 5—SUSCEPTIBILITY OF MALE AND NYMPH (THIRD INSTAR LARVAE) SPECIMENS OF *Triatoma infestans* TOWARDS TOPICALLY APPLIED DDT*

| Dosage ($\mu$g) | 50 | 100 | 500 | 800 |
|---|---|---|---|---|
| Males | 60 | 80 | 100 | 100 |
| Nymphs | 0·0 | 10 | 20 | 30 |

* Estimated as percentage killed after 120 hr of exposure.

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specimens. On account of this difference in susceptibility towards DDT, it was considered of interest to compare the activity of glycolytic and pentose phosphate pathway enzymes of nymph and male specimens. Table 6 shows the results of such a comparison. Although the glycolytic activity of nymph extracts is lower than

TABLE 6—COMPARISON OF ANAEROBIC GLYCOLYTIC RATE AND GLYCOLYTIC AND PENTOSE PHOSPHATE PATHWAY ENZYMES ACTIVITIES OF MALE AND NYMPH EXTRACTS (FRACTION II) OF *Triatoma infestans*. THE FIGURES REPRESENT ACTIVITY LEVELS UNDER SUBSTRATE SATURATION CONDITIONS OF ASSAY IN TERMS OF $\mu$MOLES OF SUBSTRATE $\times 10^2$ CONVERTED PER MINUTE PER mg N

| Activity | Males | Third instar larvae (nymphs) |
|---|---|---|
| Anaerobic glycolysis | 4·10 | 2·70 |
| Hexokinase | 13·30 | 7·20 |
| G-6-P dehydrogenase | 4·80 | 2·25 |
| 6-PG dehydrogenase | 1·55 | 0·75 |
| Aldolase | 8·95 | 7·10 |
| GAP dehydrogenase | 45·00 | 33·00 |
| Gly-P dehydrogenase | 28·80 | 19·70 |
| Phosphopyruvic kinase | 27·00 | 8·90 |
| Lactic dehydrogenase | 0·16 | 0·10 |
| Phosphopentose isomerase | 45·00 | 52·00 |
| Ribokinase | 42·50 | 24·20 |

of male extracts, in general, the activity of the individual enzymes in both nymphs and males is well above the rate of glycolysis, G-6-P dehydrogenase activity being roughly proportional to the rate of glycolysis. Only 6-PG dehydrogenase activity appears lower than the overall glycolytic rate. However, phosphopentose isomerase and ribokinase are in excess of the apparently required amounts. This would

suggest that 6-PG dehydrogenase and probably G-6-P dehydrogenase could be rate-limiting steps for reactions entering the pentose phosphate pathway. It is interesting to note that, in spite of the lower glycolytic rate of nymphs as compared with males, aldolase is approximately of the same order of magnitude in both cases. As would be expected from the data of Table 1, lactic dehydrogenase activity is negligible compared with the activity of the other assayed enzymes, while Gly-P dehydrogenase is in larger amounts than are apparently required. Apparently,

TABLE 7—THE EFFECT OF TOPICALLY APPLIED DDT AND DDE ON THE ANAEROBIC GLYCOLYTIC ACTIVITY OF CELL-FREE EXTRACTS OF MALE AND NYMPH (THIRD INSTAR LARVAE) SPECIMENS OF *Triatoma infestans*. DDT OR DDE WERE APPLIED AT THE DOSES OF 300 μg PER SPECIMEN. THE EXPOSURE PERIODS WERE AS INDICATED. THE RATIOS SHOWN WERE OBTAINED FROM FIGURES EXPRESSED IN TERMS OF μMOLES OF METABOLITE PRODUCED PER mg N PER 60 min, RELATIVE TO ACTIVITY IN CONTROL SPECIMENS, SET TO 100. THE FIGURES AFTER THE ± SIGN CORRESPOND TO THE STANDARD ERROR OF THE MEAN. CONDITIONS OF ASSAY AS IN TABLE 1

| Time of exposure (hr) | | 22 | 48 | |
|---|---|---|---|---|
| | | + Δ-pyruvate | + Δ-pyruvate | + Δ-Gly-P |
| Males | Control | 100 ± 12 | 100 ± 13 | 100 ± 11 |
| | DDT | 56 ± 13 | 90 ± 23 | 85 ± 2 |
| | DDE | 85 ± 9 | 90 ± 17 | 105 ± 12 |
| Nymphs | Control | 100 ± 19 | 100 ± 16 | 100 ± 3 |
| | DDT | 63 ± 8 | 91 ± 12 | 90 ± 12 |
| | DDE | 59 ± 23 | 111 ± 14 | 98 ± 15 |

the different susceptibility of males and nymphs would not be related to enzyme content of male and nymph specimens, since nymphs, which are more resistant than males, have, in general, a lower enzyme content than males (Table 6). However, in order to explain this different susceptibility, the effect of DDT as well as DDE on glycolytic and pentose phosphate enzymes was studied, with the hope of finding a differential sensitivity of male and nymph enzymes towards DDT. When the rate of glycolysis of Fraction II derived from male or nymph specimens exposed for 22 hr to 300 μg of either DDT or DDE was measured by the pyruvate produced, it was found that DDT significantly inhibited the pyruvate production by male extracts, from 31 to 57 per cent, while DDE only inhibited the pyruvate production from 6 to 24 per cent. However, DDT also inhibited pyruvate production in nymph extracts from 29 to 46 per cent, while the inhibition produced by DDE ranged from 18 to 64 per cent (Table 7). When the effect of DDT and DDE was studied after 48 hr of exposure to the insecticides, it was surprising to find that, instead of the inhibition of pyruvate production being more pronounced, the values were not significantly different from the pyruvate produced by control

male and nymph extracts (Table 7). Since this was a rather disturbing finding, the rate of glycolysis was also measured by the production of Gly-P after 48 hr of exposure to 300 $\mu$g of DDT or DDE. Under these conditions, slight inhibition of Gly-P production was found only in males exposed to DDT (Table 7), while no inhibition was found in nymphs treated with either DDT or DDE. This observation suggests that Gly-P dehydrogenase may be a sensitive enzyme in

TABLE 8—THE EFFECT OF DDT AND DDE ON THE ACTIVITY OF GLYCOLYTIC AND PENTOSE PHOSPHATE PATHWAY ENZYMES OF ADULT MALE AND NYMPH (THIRD INSTAR LARVAE) *Triatoma infestans* SPECIMENS. THE FIGURES REPRESENT ACTIVITY LEVELS RELATIVE TO ACTIVITY IN CONTROL SPECIMENS (SET TO 100). THE FIGURES REPRESENT THE AVERAGES OF AT LEAST FIVE EXPERIMENTS. THE FIGURES AFTER THE ± SIGN CORRESPOND TO THE STANDARD ERROR OF THE MEAN. THE INSECTS WERE TOPICALLY EXPOSED TO 300 $\mu$g OF DDT OR DDE DURING 48 hr

| | Males | | | Nymphs | | |
|---|---|---|---|---|---|---|
| | Control | DDT | DDE | Control | DDT | DDE |
| Hexokinase | 100 ± 19 | 48 ± 7 | 72 ± 13 | 100 ± 28 | 107 ± 2 | 100 ± 20 |
| G-6-P dehydrogenase | 100 ± 13 | 53 ± 12 | 83 ± 18 | 100 ± 16 | 57 ± 8 | 95 ± 17 |
| 6-PG dehydrogenase | 100 ± 16 | 42 ± 7 | 66 ± 8 | 100 ± 21 | 67 ± 19 | 97 ± 2 |
| Aldolase | 100 ± 10 | 69 ± 11 | 117 ± 6 | 100 ± 18 | 73 ± 1 | 80 ± 20 |
| GAP dehydrogenase | 100 ± 9 | 67 ± 11 | 90 ± 17 | 100 ± 4 | 107 ± 17 | 92 ± 7 |
| Gly-P dehydrogenase | 100 ± 12 | 67 ± 10 | 84 ± 25 | 100 ± 13 | 127 ± 28 | 109 ± 3 |
| Phosphopyruvic kinase | 100 ± 17 | 65 ± 7 | 67 ± 22 | 100 ± 21 | 119 ± 16 | 125 ± 30 |
| Ribolysis | 100 ± 24 | 50 ± 4 | 89 ± 28 | 100 ± 18 | 75 ± 12 | 120 ± 7 |
| Phosphopentose isomerase | 100 ± 25 | 71 ± 14 | 89 ± 19 | 100 ± 23 | 99 ± 10 | 100 ± 5 |

males and rather insensitive to DDT in nymphs. In order to verify this possibility, several of the glycolytic and pentose phosphate enzymes of Fraction II derived from male and nymph specimens, exposed for 48 hr to 300 $\mu$g of DDT or DDE, were assayed. The results obtained are shown in Table 8. With the probable exception of phosphopentose isomerase activity, all the enzymes assayed were significantly inhibited in male extracts by DDT after 48 hr of exposure to the insecticide, while in all cases the inhibition produced by DDE was not significant from the statistical standpoint. On the other hand, only aldolase and G-6-P dehydrogenase were clearly inhibited by DDT in nymphs after 48 hr of exposure to DDT (Table 8), while non-significant inhibitory effects were shown by DDE.

## DISCUSSION

Cell-free preparations obtained from thoraces and legs of various developmental stages of *T. infestans* are capable of glycolysing glucose with the production of pyruvate and Gly-P end-products (Table 1). Lactic acid formation is negligible. Although our material is not exactly comparable to that of Chefurka (1954), it is interesting to note that the amount of glucose phosphorylated by housefly extracts is not in a stoichiometric relationship, according to Chefurka's data (1954), with the lactate produced, suggesting that also in housefly another end-product, in

addition to lactate, accumulates. In view of our present results, this other end-product could be Gly-P. It should be pointed out that during anaerobic glycolysis in insect tissues, especially muscle tissue (Chefurka, 1958; Kubĭsta, 1958), the production of Gly-P has been reported. Our material corresponds essentially to muscle tissue, thus confirming the occurrence of this fermentation type in insect tissues. The explanation for this peculiarity seems to be a lacking or very weakly active lactic dehydrogenase (Table 6). Although added HDP appears to be required for optimal glycolysis, the effect of its omission could be partially reversed by increasing the ATP concentration of the medium (Table 2). This observation supports Chefurka's hypothesis (1954) that during insect glycolysis, dephosphorylative reactions compete with phosphorylative reactions, especially at the level of ATP hydrolysis and glucose phosphorylation. However, fluoride was not required for glucose phosphorylation in our system, whereas housefly extracts were not capable of phosphorylating glucose in the absence of fluoride (Chefurka, 1954).

Barron & Tahmisian (1948) reported that IAA failed to inhibit lactic acid production in teased leg muscles from *Periplaneta americana*. This lack of inhibition of lactic acid production was later on explained by Chefurka (1954) on the basis of his work on housefly, where IAA does not inhibit GAP dehydrogenase. The danger of extrapolating results obtained with one species of insect to another is obvious in view of the results obtained with *T. infestans*. *T. infestans* glycolysis (Table 2) and GAP dehydrogenase (Table 4) are sensitive to IAA. This discrepancy is not related to IAA concentration, since *T. infestans* GAP dehydrogenase is affected by concentrations lower than the one affecting the housefly enzyme, $1 \times 10^{-4}$ M (Chefurka, 1954). It is possible that this difference in sensitivity towards IAA between the housefly and *T. infestans* enzymes indicates that, in spite of the fact that both enzymes catalyse the same reaction, they may be structurally different. Supporting this possibility is the absolute requirement for added $-SH$ groups shown by the housefly enzyme, while the *T. infestans* enzyme is quite active in the absence of $-SH$ groups (Fig. 4). Apparently the enzymatic differences between housefly and *T. infestans* preparations are not limited to GAP dehydrogenase. Thus Gly-P dehydrogenase is $DPN^+$-dependent in *T. infestans* (Fig. 5), while $TPN^+$ and $DPN^+$ are both active with the housefly enzyme (Chefurka, 1954).

Although not every single step of the pentose phosphate pathway was assayed, the occurrence of this metabolic sequence appears to be rather well supported. The initial steps, hexokinase, G-6-P and 6-PG dehydrogenase, are present in cell-free extracts; R-5-P is isomerized to Ru-5-P, with concomitant production of hexoses. However, the quantitative participation of this pathway in the carbohydrate metabolism of *T. infestans* would seem to be minor, since at least 6-PG dehydrogenase is apparently a limiting step for substrates entering the pentose phosphate pathway (Table 6).

The experiments reported here demonstrate that both glycolysis and the pentose phosphate pathway are involved in the degradation of glucose in *T. infestans*. Non-phosphorylative glucose oxidation may not play a role in this connexion, since no enzymes for this pathway were found. This is in contrast

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to Sacktor's (1955) findings in the housefly, where direct glucose oxidation apparently occurs. Our results show at least quantitative differences in the carbohydrate metabolism of the various developmental stages of *T. infestans*. Glycolysis activity is very low in larvae and eggs; nymphs glycolyse to a lesser extent than males, and females glycolyse less than males and nymphs (Tables 1, 2 and 6). Differences can be established also for glycolytic and pentose phosphate pathway enzymes of males and nymphs (Table 6) and for ATP-ases (Table 3). It is possible that these differences may correspond to differences in the protein-enzymes involved rather than to conditions of the assay. This assumption is supported by the fact that GAP dehydrogenase from males and nymphs, although catalysing similar reactions, are not identical with each other. Thus, an antiserum prepared against male GAP dehydrogenase markedly inhibits the male enzyme, while not affecting the nymph enzyme; the antiserum against nymph GAP dehydrogenase only inhibits the nymph enzyme (unpublished data of the authors).

DDT clearly inhibited anaerobic glycolysis in males, after 22 hr of exposure to the insecticide. However, the fact that pyruvate production was restored to normal values after 48 hr of exposure and probably only a slight inhibition of Gly-P production persisted, make it rather doubtful that glycolytic enzymes are the primary point of attack of DDT (Table 7). Furthermore, DDE, a non-toxic analog, also inhibited pyruvate production after 22 hr of exposure. In the light of our data it is difficult to ascertain the mechanisms of action of DDT. But one fact appears more or less clear: the effect of DDT on males is different from the effect on nymphs. Some enzymes that are inhibited by DDT in males are not affected in nymphs. The possibility that these differences might be explained in terms of different rates of penetration of DDT into male and nymph specimens cannot be completely ruled out at present. We know that approximately 58 per cent of topically applied $C^{14}$ – DDT is absorbed in nymphs after 48 hr exposure, but comparative data in the case of male specimens are not yet available (unpublished data of the authors). It is also possible that the lack of inhibition of several of the glycolytic and pentose phosphate pathway enzymes in nymphs after 48 hr of exposure may be explained by a faster excretion of DDT in nymphs than males or by a more efficient detoxifying mechanism in nymphs than males, factors which would also explain the lesser susceptibility of nymphs. In any case, our results seem to indicate that differential enzymatic inhibition may be involved in the mechanism of action of DDT and probably is related to the mechanism of resistance towards DDT.

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## BOOK REVIEW

M. A. GEREBTZOFF: **Cholinesterases. A Histochemical Contribution to the Solution of Some Functional Problems.** Pergamon Press, London, 1959. vi + 195 pp. £2 15s.

As indicated on the monograph's inner cover, the histochemical research on which this publication is based "represents six years of team work in the Department of Anatomy Laboratory of Liège University". The reader should be aware of this definition of scope, which is reflected also in the title, inasmuch as during the past decade a tremendous volume of literature has also been published from several other laboratories on the same subject. While the latter work is surveyed in the monograph in a representative fashion, it is assigned to a secondary position. This should not be construed as criticism. To have provided equal coverage of all the literature in the immediate field would have required a volume of considerably greater size and cost.

Dr. Gerebtzoff and his associates have been extremely productive in surveying histochemically the distributions of cholinesterases (ChE's) in practically all the important systems, including the central nervous system, in various mammalian and lower species, and in some cases at various stages of embryological development. Throughout the monograph, an attempt is made to correlate histochemical findings with known physiological function, but the emphasis is primarily morphological.

The chief point of criticism which should be mentioned relates to methodology, and the consequent results and conclusions drawn. Essentially all the work reported from the

Liège laboratory has been done by a modification of the thiocholine ester method which has included formalin fixation at two steps, before and after sectioning. Enzymatic histochemistry inevitably presents the problem of seeking optimal preservation of morphological detail with minimal loss of enzymatic activity. Formalin fixation favors the former at the expense of the latter. Moreover, as Couteaux and his associates have shown, the degree of inactivation of both acetylcholinesterase (AChE) and non-specific cholinesterase (ChE) by formalin is extremely variable and is dependent not only upon the concentration of the fixative, thickness of the tissue, and time of exposure, but on the nature of the structures with which the enzymes are associated. Couteaux, who first applied formalin fixation to the thiocholine ester method, and with highly successful results, has recommended that it be employed only in parallel with unfixed, fresh-frozen sections, so as to avoid the danger of inactivating completely the enzymes at certain sites (*Exp. Cell Res.* Suppl. **5**, 294, 1958). The results of the failure to do so in the present work are exemplified by statements regarding findings with cholinergic motor nerve fibres: "But a general rule prevails: the motor nerve fibre has no AChE activity" (p. 49), and: "We must insist on the fact that the presynaptic localization . . . (of AChE) . . . is found, in motor fibers, only in the most primitive vertebrates" (p. 50). In the reviewer's laboratory, AChE has been found consistently in such fibers both histochemically, with fresh-frozen sections, and by quantitative determinations on homogenates of motor nerves. Consequently, other negative findings reported in the present monograph may be open to question.

A less serious objection, for which justification can be found, is the fact that the method appears to have been tailored, with respect to incubation time and pH, for each tissue in order to obtain a fairly uniform intensity of staining of all sites of enzymatic activity. Admittedly, microscopic histochemistry is "roughly quantitative" in only the crudest sense. Nevertheless, a general impression of relative intensities of enzymatic activity, which are of great physiological significance, can be obtained when conditions of staining are kept constant. Since the legends accompanying all photomicrographs state the conditions of staining, the reader has the opportunity to deduce the relative activities represented, but with two variables involved this can be misleading.

In spite of the foregoing limitations, Dr. Gerebtzoff's monograph is an extremely well prepared summary of a great amount of important work which would otherwise be very difficult for the reader to assemble from the original sources. It should be considered an essential item for any investigator in the field covered.

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# HOST–PARASITE RELATIONSHIPS IN ECHINOCOCCOSIS—IV. ACETYLCHOLINESTERASE AND PERMEABILITY REGULATION IN THE HYDATID CYST WALL*

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**Abstract**—(1) A specific acetylcholinesterase is present in homogenates of hydatid scolices and brood capsules of *Echinococcus granulosus*. The rate of hydrolysis of acetylcholine is 45 times that of butyrylcholine. Enzyme activity is partially inhibited by physostigmine and hexaethyltetraphosphate (HETP).

(2) The reducing substance ("glucose") contents of fluids of 22 hydatid cysts of the bovine lung ranged from 9·2–102·5 mg per cent, of 24 cysts of the bovine liver, from 13·9–101·1 mg per cent. The cyst fluid "glucose" level is higher in cysts from sheep than in cysts from cattle.

(3) In hydatid daughter cysts suspended in isotonic Krebs–Ringer's solution for 4 hr, the outward passage of "glucose" occurs very slowly. Passage is accelerated by the addition of small amounts of certain acetylcholinesterase inhibitors to the suspending solution and markedly accelerated by the addition of both HETP and either choline or acetylcholine.

(4) Accelerated outward passage of "glucose" also occurs when daughter cysts are suspended in hypotonic salt solutions of 0·04 M concentration or less.

(5) The weight changes of hydatid daughter cysts suspended in a variety of salt solutions of from 0·039 M to 0·616 M for 2·5 hr are slow, regular and predictable. In solutions similar chemically but more hypotonic, permeability control in the cyst wall appears to break down following a period of water imbibition at the expected rate. Acetylcholine chloride (0·01 M) maintains permeability control to a greater degree than does NaCl at the same molar concentration.

(6) Hydatid daughter cysts lose weight more rapidly when suspended in a hypertonic glucose–saline solution for 3 hr than they do in the same glucose–saline which contains small amounts of acetylcholinesterase inhibitors.

(7) Extracts of hydatid cyst fluid and hydatid tissues contain one or more unidentified quaternary ammonium compounds.

## INTRODUCTION

WITHDRAWAL of the germinal membrane from the laminated membrane occurs slowly in *Echinococcus granulosus* daughter cysts immersed in certain hypertonic solutions which contain potassium ion. Separation of the two membranes under these conditions is partially prevented by the addition to the suspending medium

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of small amounts of acetylcholine, physostigmine or iodoacetate (Schwabe, 1959). These observations suggested the possibility that an acetylcholinesterase system functions in permeability control in the hydatid cyst wall (HCW). The present paper is concerned with the demonstration of specific acetylcholinesterase in hydatid tissues and with further evidence that it may function in permeability control and osmoregulation.

## MATERIALS AND METHODS

### *Manometric methods*

Scolices and brood capsules of *E. granulosus* were obtained from bovine cysts (Farhan *et al.*, 1959) and washed several times by suspension and slow centrifugation in buffer solution. Homogenates were prepared in ice-cold bicarbonate buffer (Nachmansohn & Rothenburg, 1945) with a Teflon-glass tissue grinder. Aliquots of the homogenate were reduced to constant weight at 108°C and corrected dry weights obtained by subtracting the weight of the salts present in the suspending buffer. Acetylcholinesterase activity was determined in 6 ml Warburg flasks with a single sidearm according to the procedure of Bueding (1952). Buffered substrate, or a buffered mixture of substrate and inhibitor, was tipped in following temperature equilibration. Appropriate corrections were made for gas exchange by the homogenate without substrate and for the reaction mixture without homogenate. Enzymatic activity was expressed as $mm^3$ of $CO_2$ liberated per mg dry weight of tissues during the first 15 min following temperature equilibration. Enzymatic activity remained constant for at least the first 20 min. A mixture of 5 per cent $CO_2$ and 95 per cent $N_2$ was prepared from cylinder gases by displacement of water. Moisture and oxygen were removed by passing the gas mixture through $CaCl_2$ or Drierite and heated copper turnings, respectively. Bueding (1952) has reported the optimum concentration of acetylcholine for other helminth tissues as 0·04 M.

### *Permeability measurements*

Hydatid daughter cysts (HDC) of *E. granulosus* were obtained and the experiments on weight changes performed as described previously (Schwabe, 1959). For the experiments in which passage of "glucose" was followed, HDC, most of which weighed 3–4 g, were washed quickly in isotonic Krebs–Ringer's solution (KRS). Washed cysts were added to 25 ml graduated cylinders containing 10 ml of suspending medium each, until the total volumes (medium plus cysts) were 15 ml. This usually required one 3–4 g cyst and one or two small cysts per cylinder. By using equal volumes of cysts and giving as much attention to size and numbers of cysts as the uniformity of the material permitted, a reasonably close approximation of surface areas was achieved. Cylinders were immersed in a constant temperature water bath at 37°C. Solutions were immediately agitated with a pipette and a 5 ml aliquot withdrawn. This was promptly replaced by 5 ml of fresh suspending solution. Another 5 ml aliquot was removed after 2 hr incubation; this was not replaced. After 4 hr, the remaining suspending fluid

was withdrawn. These samples were stored in serum vials at −30°C until the chemical determinations were run.

Reducing substances in the suspending solution aliquots were determined in duplicate by the Simmons & Gentzkow (1955) modification of Nelson's method and expressed as "glucose". Preparation of protein-free filtrates (as for determination of blood glucose) diluted most samples too highly for accurate measurements. Since there was no turbidity and as little, if any, protein could be demonstrated in 4-hr samples with Biuret reagent, this step was omitted. KRS (prepared according to Umbreit *et al.*, 1957, p. 149) is isotonic with rat serum; it contains NaCl, KCl, $CaCl_2$, $KH_2PO_4$ and $MgSO_4$.

*Paper chromatographic methods*

Hydatid tissues were extracted and quaternary ammonium bases isolated as reineckates by a slight modification of the procedure of Augustinsson & Grahn (1953). Chromatograms were run with n-butanol–ethanol–acetic acid–water (8:2:1:3) and developed by spraying with dipicrylamine (Augustinsson & Grahn, 1953), hydroxylamineferric chloride (Whittaker & Wijesundra, 1952), potassium iodoplatinate (Mannering *et al.*, 1954) and potassium iodobismuthite (Sandri, 1953). The latter reagent, originally proposed for the microdetection of betaine, was slightly modified for this purpose by first dissolving 1·5 g bismuth subcarbonate in 30 ml concentrated HCl; 150 ml of distilled water were added and the resulting solution mixed with 30 g of KI in 80 ml distilled water. To each 50 ml of this mixture was finally added 0·2 ml of a 2 per cent iodine, 5 per cent potassium iodide solution.

*Chemicals*

Chemicals were of reagent grade. Butyrylcholine chloride and hexaethyltetraphosphate (HETP) were obtained from Bios Laboratories, New York; the other biochemicals from Nutritional Biochemical Company, Cleveland, Ohio. According to Whittaker (1951), HETP is a mixture of ethylpolyphosphates, the active principle of which is tetraethylpyrophosphate.

## RESULTS

*Acetylcholinesterase in hydatid tissues*

Manometrically, specific acetylcholinesterase activity was demonstrated in homogenates of hydatid scolices and brood capsules (Table 1). The mean rate of hydrolysis of acetylcholine chloride was 45 times that of butyrylcholine chloride. Enzyme activity was inhibited 54 per cent by 0·001 M HETP, 85 per cent by 0·002 M HETP, 32 per cent by 0·001 M physostigmine salicylate and 86 per cent by 0·002 M physostigmine salicylate. These inhibition rates were not as high as those often reported for acetylcholinesterases of other tissues.

*Outward passage of reducing substances ("glucose") through the hydatid cyst wall*

Glucose was reported by a number of early investigators to be a constituent of HCF. Recent indirect evidence indicated that the intact HCW was permeable to

glucose when the latter was present in excess in the cyst's external environment (Schwabe, 1959). Preliminary experiments showed also that the "glucose" (more correctly, reducing substances) of HCF would pass slowly through the intact HCW from inside to outside when HDC were suspended in balanced isotonic

TABLE 1—ACETYLCHOLINESTERASE ACTIVITY ($mm^3$ $CO_2$/mg dry wt./15 min) OF HOMOGENATES OF HYDATID SCOLICES AND BROOD CAPSULES

| Experiment No. | 0·04 M Acetylcholine chloride | 0·04 M Butyrylcholine chloride | 0·04 M Acetylcholine chloride, 0·001 M HETP | 0·04 M Acetylcholine chloride, 0·001 M physostigmine salicylate | 0·04 M Acetylcholine chloride, 0·002 M HETP | 0·04 M Acetylcholine chloride, 0·002 M physostigmine salicylate |
|---|---|---|---|---|---|---|
| 1 | 6·60 | 0·21 | — | — | — | — |
| 2 | 6·25 | 0·02 | — | — | — | — |
| 3 | 6·80 | — | 2·20 | — | — | — |
| 4 | 5·15 | — | 2·80 | 3·70 | — | — |
| 5 | 5·65 | — | — | — | 0·91 | 1·28 |
| 6 | 2·20 | — | — | — | 0·67 | 0·25 |
| $\bar{X}$ | 5·44 | 0·12 | 2·50 | 3·70 | 0·79 | 0·77 |

salt solutions. While these observations suggested an experimental situation in which to study permeability control in the HCW, it was necessary to establish first that "glucose" was a constant constituent of HCF of bovine origin.

The fluids of 62 hydatid cysts, collected by syringe from bovine lungs and livers 2–4 hr following slaughter, were filtered through paper to remove brood capsules and scolices and quickly frozen and stored in serum vials at −30°C. "Glucose" was determined in duplicate in a Klett–Summerson photoelectric colorimeter by the method of Nelson as modified by Simmons & Gentzkow (1955). Protein-free filtrates were prepared by the barium hydroxide–zinc sulfate method. "Glucose" levels, grouped according to host organ and cyst characteristics are given in Tables 2 and 3 (a few determinations on HCF from sheep and man are also included). The large standard deviations indicate the variation encountered from cyst to cyst (the range in 22 fertile cysts of the bovine lung was 9·2–102·5 mg per cent; in 24 fertile cysts of the bovine liver, 13·9–101·1 mg per cent). The differences in mean values do suggest, however, that the HCF "glucose" levels of healthy, fertile cysts are perhaps higher than those of non-fertile or decomposing cysts, and also that HCF "glucose" levels are higher in sheep than in cattle. Only that difference between species is considered statistically significant, however

(Student's *t* test, *P* of less than 0·02). A further series of "glucose" determinations on the fluids of 80 HDC from cattle gave a mean level of 36·30±22·22 mg per cent.

Normal serum glucose levels for local cattle and sheep are not available at present and we were not able to secure blood samples from known infected animals at the abattoir. Taking published standard serum values for comparison, it would appear that the HCF glucose ranges are somewhat greater but that the HCF

TABLE 2—REDUCING SUBSTANCE ("GLUCOSE") LEVELS (mg %) IN HYDATID CYST FLUIDS ACCORDING TO HOST ORGAN AND CYST FERTILITY

| | Lung | Liver |
|---|---|---|
| Scolices numerous and motile | 52·4 ± 29·1 (22)* Bovine<br>80·8 ± 24·7 (7) Ovine<br>71·6 (1) Human | 53·9 ± 24·6 (24) Bovine<br>96·9 ± 8·7 (6) Ovine<br>42·2 ± 20·2 (2) Human |
| Scolices absent, few or degenerated | 33·5 ± 26·3 (13) Bovine | 39·4 ± 18·3 (3) Bovine |

* Mean ± standard deviation; numbers in parentheses in tables, figures and text indicate the number of cysts or the number of experiments in each instance.

TABLE 3—REDUCING SUBSTANCE ("GLUCOSE") LEVELS (mg %) IN HYDATID CYST FLUIDS ACCORDING TO HOST ORGANS AND CONDITION OF CYST MEMBRANES*

| | Lung | Liver |
|---|---|---|
| Membranes apparently healthy, white and intact | 51·2 ± 32·0 (9) Bovine | 61·1 ± 13·4 (15) Bovine<br>42·2 ± 20·2 (2) Human |
| Membranes thin, yellow or degenerated | 43·3 ± 8·0 (8) Bovine | 46·3 ± 26·6 (7) Bovine |

* The number of HCF values in Table 3 are fewer than in Table 2 because observations on the condition of cyst membranes were not recorded for the first group of cysts examined.

mean values are lower than those for the respective sera. Coudounis & Polydoridès (1936) reported, also, that in each of five comparisons made, the blood sugar level of operated human patients was somewhat higher than that of the corresponding HCF.

In a few experiments in which we have incubated at 37°C Seitz-filtered HCF and aseptically-collected, centrifuged HCF (scolex-free), there was no change in "glucose" level during a 24-hr period. Hydatid tissue glycolytic mechanisms have recently received attention by Agosin & Aravena (1959).

The outward passage of "glucose" from HCF through the HCW into various suspending media was measured during a 4-hr period. These media included KRS and KRS containing respectively 0·01 M iodoacetate, 0·01 M physostigmine

salicylate, 0·01 M HETP, 0·01 M atropine sulfate, 0·01 M acetylcholine chloride, 0·04 M acetylcholine chloride plus 0·01 M HETP, 0·04 M choline chloride plus 0·01 M HETP and, in addition, distilled water, 0·01 M NaCl, 0·04 M NaCl,

TABLE 4—GLUCOSE (mg %) PRESENT IN SUSPENDING MEDIA FOLLOWING INCUBATION OF EQUAL VOLUMES OF HYDATID CYSTS AT 37°C

| | Suspending medium | Incubation time (hr) | | | Permeability control |
|---|---|---|---|---|---|
| | | 0 | 2 | 4 | |
| Isotonic | (*A*) KRS (26) | 0·55 ± 0·62 | 0·66 ± 0·63 | 0·93 ± 0·87 | + + |
| | (*B*) KRS + 0·01 M Acetylcholine chloride (9) | 0·32 ± 0·36 | 0·46 ± 0·37 | 0·52 ± 0·32 | + + |
| | (*C*) KRS + 0·01 M Iodoacetate (7) | 0·48 ± 0·67 | 1·04 ± 0·53 | 6·93 ± 3·80 | ± (I) |
| | (*D*) KRS + 0·01 M Atropine $SO_4$ (6) | 0·79 ± 0·44 | 2·93 ± 1·25 | 5·56 ± 0·87 | ± (I) |
| | (*E*) KRS + 0·01 M HETP (9) | 0·07 ± 0·00 | 2·59 ± 1·30 | 4·90 ± 1·44 | ± (I) |
| | (*F*) KRS + 0·01 M HETP + 0·04 M Acetylcholine chloride (6) | 0·54 ± 0·30 | 9·94 ± 4·16 | 14·34 ± 3·42 | − (I) |
| | (*G*) KRS + 0·01 M HETP + 0·04 M Choline chloride (6) | 0·64 ± 0·35 | 5·87 ± 2·16 | 10·68 ± 3·84 | − (I) |
| Hypotonic | (*H*) Distilled water (7) | 0·07 ± 0·07 | 2·59 ± 1·39 | 6·59 ± 2·96 | ± (D) |
| | (*I*) 0·01 M NaCl (3) | 0·70 ± 0·56 | 3·58 ± 1·24 | 7·40 ± 1·62 | ± (D) |
| | (*J*) 0·01 M Acetylcholine chloride (6) | 0·70 ± 0·73 | 2·91 ± 1·00 | 6·90 ± 2·40 | ± (D) |
| | (*K*) 0·01 M Choline chloride (4) | 0·17 ± 0·17 | 4·33 ± 1·42 | 7·80 ± 1·22 | ± (D) |
| | (*L*) 0·04 M NaCl (15) | 0·23 ± 0·27 | 0·81 ± 0·68 | 1·45 ± 0·84 | + |
| | (*M*) 0·04 M Acetylcholine chloride (10) | 0·39 ± 0·48 | 1·11 ± 0·66 | 1·90 ± 0·82 | + |
| | (*N*) 0·04 M Choline chloride (14) | 0·69 ± 1·16 | 2·94 ± 3·72 | 7·33 ± 6·68 | ± (D) |

+ + Complete control, + good control, ± poor control, — no control, (I) acetylcholinesterase inhibitor, (D) possible dialysis effect.

0·01 M choline chloride, 0·04 M choline chloride, 0·01 M acetylcholine chloride, 0·04 M acetylcholine chloride and 0·01 M NaCN. Physostigmine salicylate and NaCN interfered with the determinations of "glucose"; the other results are shown in Table 4.

While results for each suspending medium are expressed in Table 4 in terms of a mean value and its standard deviation, they represent the pooled results of more than one experiment in each of which the medium was run against its appropriate control, e.g. KRS versus KRS plus 0·01 M iodoacetate.

With 36·20 mg per cent as the mean "glucose" content of fluids of HDC of bovine origin, the theoretical concentration in the suspending medium at equilibrium under these experimental conditions would be approximately 12 mg per cent. Approximations of this theoretical mean concentration were attained at 4 hr in HDC suspended in KRS containing 0·01 M HETP plus either 0·04 M acetylcholine or 0·04 M choline. In these two suspending media, the outward passage of "glucose" was particularly rapid, whereas in KRS alone and KRS containing 0·01 M acetylcholine chloride, "glucose" passage occurred very slowly. The differences between those 4-hr "glucose" levels for suspending media designated as maintaining complete, good, partial or poor permeability control (Table 4) are in most instances statistically significant ($P$ of less than 0·01, Student's $t$ test). These include $\triangle_{C-A}$, $\triangle_{C-M}$, $\triangle_{E-M}$ and $\triangle_{G-E}$. Non-significant differences not obvious upon inspection are $\triangle_{G-C}$ and $\triangle_{L-A}$.

*Passage of water through the hydatid cyst wall*

In a previous paper (Schwabe, 1959), data were presented on weight changes in HDC suspended in solutions of varied tonicity and chemical composition. From these data it was possible to conclude that the HCW was permeable in both directions to water and permeable from without to within, at least, to several electrolytes and non-electrolytes. The latter arranged themselves in order of molecular weights. In addition, in certain hypotonic suspending solutions the permeability control mechanism of the HCW appeared to break down following a period of water imbibition at the expected rate. It was suggested that some water-soluble substance essential to permeability control was being dialysed from the HCW in hypotonic media.

These experiments were continued and, to date, weight changes have been determined over a 2·5 hr incubation period for 229 HDC. Expected slow weight changes occurred in all HDC suspended in 0·039–0·616 M solutions of NaCl (26), in sheep serum (3), in sheep serum diluted 1 : 2 (3), in solutions of KCl, $KH_2SO_4$ and $MgSO_4$ isosmotic with rat serum (20), in KRS (12), and in 0·04 M solutions of acetylcholine chloride (6), choline chloride (6) and KCl (6) (Fig. 1). Permeability control in the HCW began to break down within 2·5 hr, however, in distilled water (6 out of 8); tap water (3); 0·001 NaCl (7); 0·001 M NaCl, 0·001 M KCl (8); 0·005 M NaCl (8); 0·01 M NaCl (26); 0·01 M acetylcholine chloride (25); 0·01 M choline chloride (6); 0·01 M KCl (8); HCF diluted 1 : 20 (3) and sheep serum diluted 1 : 4 (2). In sheep serum diluted 1 : 4 the breakdown in permeability control was somewhat delayed (2–2½ hr incubation) and 0·01 M acetylcholine chloride appeared to maintain permeability control to a greater extent than did choline chloride, NaCl or KCl of the same molar strength (Fig. 2). The mean weight per original mg of HDC suspended in 0·01 M NaCl for 2½ hr was $0{\cdot}98 \pm 0{\cdot}14$

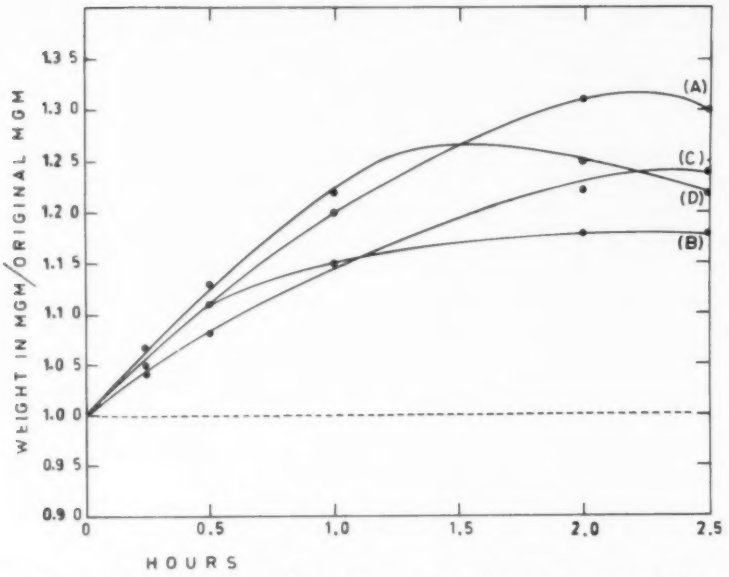


FIG. 1. Change in weight of hydatid cysts suspended in 0·04 M solutions of (A) acetylcholine chloride, (B) choline chloride, (C) NaCl, (D) KCl.

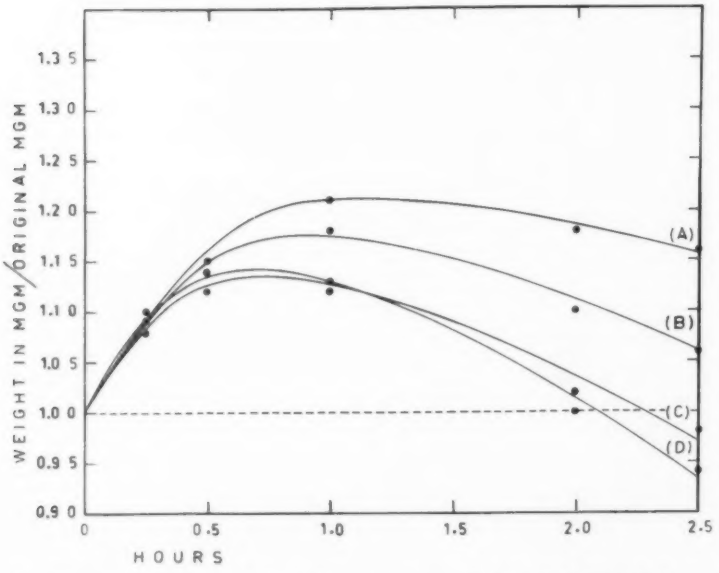


FIG. 2. Change in weight of hydatid cysts suspended in 0·01 M solutions of (A) acetylcholine chloride, (B) choline chloride, (C) NaCl, (D) KCl.

(26), in 0·01 M acetylcholine chloride it was $1{\cdot}16 \pm 0{\cdot}28$ (25). This difference is statistically significant with a $P$ of less than 0·01 (Student's $t$ test).

The mean weight per original mg of HDC suspended for 3 hr at 37°C in 0·7276 M glucose, 0·154 M NaCl was $0{\cdot}69 \pm 0{\cdot}04$ (15). Subsequent measurements made on HDC suspended in the same glucose–saline solution but containing, respectively, 0·01 M HETP, 0·01 M physostigmine salicylate and 0·01 M atropine sulfate gave mean weights per original mg of $0{\cdot}75 \pm 0{\cdot}10$ (6), $0{\cdot}78 \pm 0{\cdot}04$ (6) and $0{\cdot}76 \pm 0{\cdot}05$ (6). The differences between glucose–saline and glucose–saline plus either physostigmine or atropine are statistically significant (Student's $t$ test, $P$ less than 0·01).

Following 2·5 hr suspension in KRS plus 0·01 M HETP the mean weight per original mg of HDC was $0{\cdot}82 \pm 0{\cdot}06$ (6), KRS plus 0·01 M atropine sulfate $0{\cdot}92 \pm 0{\cdot}02$ (8) while in KRS alone $0{\cdot}941 \pm 0{\cdot}0123$ (12).

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*Chromatographic analyses of HCF and hydatid tissues for choline-related compounds*

Neither HCF nor HCF concentrated by evaporation *in vacuo* with a twenty-fold reduction in volume gave detectable spots when applied to strips in amounts up to 50 λ. In trichloracetic acid–alcohol extracts of concentrated HCF and in acetone solutions of the reineckate fraction of the extract, however, one spot appeared which stained as a quaternary ammonium base. The large number of compounds in this group precluded identification on the basis of $R_f$ value and color alone.

TABLE 5—QUATERNARY AMMONIUM COMPOUNDS DETECTED ON PAPER CHROMATOGRAMS OF HYDATID TISSUES

| | Concentrated TCA-alcohol extract | Acetone solution of reineckates | Chlorides |
|---|---|---|---|
| Pooled germinal and laminated membranes | 1–3 spots | 1–2 spots | No spots |
| Scolices and brood capsules | 1–4 spots | 1 spot | 1 spot |

In extracts of the HCW (laminated and germinal membranes together) and in extracts of hydatid scolices, isolation of quaternary ammonium compounds was indicated by the appearance of pink to yellow precipitates as insoluble reineckates. One to four spots appeared on chromatograms of the acetone solution of the reineckates and of the subsequent chloride (Table 5). These could not be reliably identified on the basis of color and $R_f$ in as much as when known amounts of choline, its esters or related compounds were added to control samples, not only did their $R_f$ values differ from those obtained in pure solutions, but frequently the number of detectable spots did not correspond to the number of added

compounds. Chromatograms of mixtures of pure solutions of quaternary ammonium compounds gave clear cut separations, however. It is likely that salts present in the materials examined were one source of difficulty. It was concluded from this merely that one or more quaternary ammonium compounds are present in extracts of hydatid tissues and of HCF.

## DISCUSSION

Although the paper chromatographic methods reported were of no value for the specific identification of acetylcholine or related compounds, the presence of one or more quaternary ammonium compounds is indicated. Lemaire & Ribère (1935) previously had reported lecithin to be a constituent of HCF and, as pointed out by Baldwin (1952), it is in this form that choline usually occurs in nature. Flössner (1924) indicated also the presence of betaine which is frequently found in association with choline. Latif & el Kordy (1946) demonstrated by chemical methods niacin in concentrations up to 350 $\mu$g per cent in HCF obtained from infected camels. Although niacin gives a distinctive color with potassium iodobismuthite, the chromatographic method was not sufficiently sensitive to detect niacin in this quantity.

The presence of specific acetylcholinesterase in helminth tissues was first demonstrated by Bueding (1952) for *Schistosoma mansoni*, *Litomosoides carinii* and *Ascaris lumbricoides*. Previously, esterases capable of hydrolysing acetylcholine had been noted in other helminths, including *Cysticercus pisiformis*, by relatively non-specific methods (von Brand, 1952). Hydatid scolices and/or brood capsules of *Echinococcus granulosus* contain the specific enzyme, while less direct evidence suggests that it occurs too in the germinal membrane. It has not yet been possible to obtain for manometric experiments usable amounts of scolex-free germinal membranes from cysts of known viability. The presence of a specific acetylcholinesterase in the two nematodes, one trematode and one cestode thus far studied suggests that this enzyme is of common occurrence in helminths.

Those effects reported here of several acetylcholinesterase inhibitors upon hydatid tissues have been of four types:

1. Interference with germinal membrane withdrawal of HDC suspended in certain hypertonic solutions which contain $K^+$.
2. More rapid outward passage of "glucose" through the HCW of HDC suspended in isotonic salt solutions.
3. Less rapid loss of water from HDC suspended in hypertonic glucose solutions, probably by allowing more rapid inward passage of glucose through the HCW.
4. Partial inhibition of acetylcholinesterase activity.

These observations suggest strongly that the acetylcholinesterase of hydatid tissues is involved in permeability control and osmoregulation.

Bueding (1952) had suggested previously that the presence of acetylcholinesterases in helminths may be related to motor activity. It is of interest to note, in this connexion, that various hydatid tissues, in addition to scolices, contain

contractile elements. Brood capsule membranes pulsate and contract vigorously. Complete eversion of brood capsules often occurs in scolex cultures following *in vitro* incubation for a day or two. The stalk attaching the scolex to the brood capsule membrane is also contractile as is the small "nucleus" of hooklet-containing tissue which persists on the germinal membrane of hydatid cysts arising from scolices inoculated intraperitoneally into mice (Schwabe *et al.*, 1959).

Though there are recent reviews on the subject, the question of the specific pharmacodynamic actions upon different tissues of the many reported cholinesterase inhibitors is rather confusing. At the present time we are observing the *in vitro* effects of various drugs upon the respiration of hydatid tissues. If the actions of any drugs suggest possible use in the chemotherapy of hydatid disease, it will be necessary then to examine their abilities to permeate the HCW, and for any drugs with reported anti-cholinesterase activities, it will be of interest to note as well the general effects upon HCW permeability.

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# THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE TISSUES OF THE FRESHWATER CRAYFISH *ASTACUS PALLIPES* LEREBOULLET

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**Abstract**—1. The non-protein nitrogenous substances of the tissues of the freshwater crayfish *Astacus pallipes* have been examined by chromatographic methods.

2. About 80 per cent of the non-protein nitrogen of abdominal muscle and 70 per cent of the non-protein nitrogen of the hepatopancreas has been accounted for.

3. With the exception of betaine all the non-protein nitrogenous substances found by Kermack *et al.* (1955) in tissues of the marine decapod *Homarus vulgaris* were found in the tissues of *Astacus pallipes*.

4. The concentrations of non-protein nitrogenous substances were much lower in the tissues of *Astacus pallipes* than in the tissues of *Homarus vulgaris*.

5. The presence of phosphorylethanolamine and of glycerylphosphorylethanolamine in the hepatopancreas of *Astacus pallipes* is reported.

## INTRODUCTION

RECENT work on the non-protein nitrogenous constituents of crustacean muscle tissue has stemmed largely from an interest in the role of small organic molecules in maintaining the osmotic balance of cells and in adjusting the osmotic pressure in the cells of euryhaline species.

The studies of Duchâteau & Florkin (1955), Robertson (1957) and Shaw (1958) have shown that large quantities of free amino acids, trimethylamine oxide and similar substances are present in the muscle tissue of marine crustaceans. Much lower concentration of these substances are found in the muscles of freshwater decapods such as *Astacus pallipes* (Camien *et al.*, 1951) and in *Eriocheir sinensis* which had been adapted to freshwater (Duchâteau & Florkin, 1955).

Kermack *et al.* (1955) examined extensively the non-protein nitrogenous constituents of the tissues of the lobster, *Homarus vulgaris*. I considered that a similar study of the non-protein nitrogenous substances of the related freshwater decapod *Astacus pallipes* would yield information of interest from a comparative point of view. The only other analysis of this animal of which I am aware, that of Camien *et al.* (1951), was concerned with muscle tissue only. These workers determined the concentrations of fifteen amino acids in acid hydrolysates of muscle extract, by microbiological assay.

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In addition, the identification of chemical intermediates in invertebrate tissues might lead to a better understanding of metabolism in these animals and might provide information on the sites of metabolic change. This in fact has proved to be so with the finding of phosphorylethanolamine and glycerylphosphorylethanolamine in the hepatopancreas of *Astacus pallipes*.

## MATERIALS AND METHODS

Crayfish (*Astacus pallipes*) were either collected from local streams or obtained from L. Haig & Co., Ltd., Dorking, Surrey. The animals were maintained in the laboratory, in running tap water. Two batches, referred to as A and B, were used.

### *Extraction*

Animals were killed by severing the ventral nerve cord; the hepatopancreas and abdominal muscle were rapidly removed from several animals, pooled and weighed. The tissues (20–30 g) were then homogenized for 5 min with twice their weight of water in an M.S.E. homogenizer. The solids were centrifuged off and re-extracted with a further three 30 ml portions of water. By this procedure more than 98 per cent of the water-soluble nitrogenous substances are extracted (cf. Kermack *et al.*, 1955). The aqueous extracts were combined and an equal volume of 10% (w/v) trichloroacetic acid was added. The precipitated protein was removed by centrifuging and the extract concentrated to about 40 ml in a rotary film evaporator. Most of the trichloroacetic acid was then removed from the extract by shaking it with five successive volumes of 200 ml diethyl ether. Glycogen was removed from the extract by adding ethanol to a concentration of 60–70 per cent and allowing the mixture to stand in a refrigerator overnight. The precipitated glycogen was removed by centrifuging. Finally the alcohol was removed by distillation under reduced pressure and the extract concentrated to a convenient volume (20–30 ml).

### *Chemical methods*

*Total N* was determined by a micro-Kjeldahl method in a Markham (1942) type apparatus. The distillate was collected in 4% (w/v) boric acid containing 1 ml of the mixed indicator of Conway & O'Malley (1942).

*α-Amino N* was determined by the method of Pope & Stevens (1939).

*Glutamine amide N, trimethylamine oxide N and volatile base N* were determined by the methods of Kermack *et al.* (1955).

*Amino acids* were separated and estimated by the method of Moore *et al.* (1958). The values for the hepatopancreas extract obtained by this procedure were complicated by the presence of several unidentified substances, presumably peptides, and as an aid in the identification of the detected amino acids paper chromatography was employed in a duplicate analysis as described by Stein & Moore (1954).

To detect the presence of *betaine*, extracts remaining from chemical analyses were concentrated, by removal of water under reduced pressure, approximately tenfold. They were then chromatographed on paper, the solvent system of Bartley (1954) and the spray reagents of Bartley (1954) and of Augustinsson & Grahn (1953) being used.

The *nitrogen* content of fractions containing glycerylphosphorylethanolamine was estimated by the method of Jacobs (1959) and that of phosphorus by the method of Fiske & Subbarow (1925).

*Hydrolysis*

To obtain information on peptide bound amino acids 1 ml of extract (see p. 174) was hydrolysed with 12 ml of 6 N-HCl in a sealed glass tube for 24 hr at 110°. After hydrolysis the contents of the tube were evaporated to a very small volume under reduced pressure; distilled water was then added and the procedure repeated several times, to remove HCl. The hydrolysate was then made to the desired volume with 0·2 N-sodium citrate, pH 2·2 (Moore & Stein, 1954). The hydrolysates were then analysed immediately.

## RESULTS

Table 1 shows the quantities of various nitrogenous substances found in extracts of the hepatopancreas and muscle of the crayfish. No betaine was found in extracts of either tissue.

TABLE 1—PARTITION OF NON-PROTEIN NITROGENOUS SUBSTANCES IN TISSUES OF *Astacus pallipes* (mg/100 g wet tissue)

| | Total N | α-Amino N | Trimethyl-amine oxide N | Amide N | Volatile base N |
|---|---|---|---|---|---|
| Muscle A | 411 | 176 | 12·3 | 15·4 | 18·0 |
| Muscle B | 411 | 150 | 22·0 | 13·0 | 9·0 |
| Hepato-pancreas A | 379 | 148 | 5·0 | 19·0 | 13·0 |
| Hepato-pancreas B | 388 | 123 | 5·0 | 14·5 | 13·7 |

Table 2 shows the quantities of amino acids and other ninhydrin positive substances found in the tissue extracts examined. Muscle B and hepatopancreas B were analysed in duplicate.

Some 80 per cent of the water-soluble non-protein nitrogenous constituents of muscle are accounted for as 35 per cent α-amino N, 31 per cent amino acid N

(other than α-amino N), 5·4 per cent trimethylamine oxide N, 3·1 per cent amide N, 2·2 per cent taurine N and 2·1 per cent volatile base N.

Of the water-soluble non-protein nitrogenous substances in the hepatopancreas about 70 per cent are accounted for as 33 per cent α-amino N (after hydrolysis of the

TABLE 2—AMINO ACIDS AND RELATED SUBSTANCES IN TISSUES OF *Astacus pallipes*
(Values per 100 g wet wt. tissue)

| | Muscle | | | | Hepatopancreas | | | |
|---|---|---|---|---|---|---|---|---|
| | Unhydrolysed | | Hydrolysed | | Unhydrolysed | | Hydrolysed | |
| | (mM) | α-$NH_2$ (N mg) | (mM) | α-$NH_2$ (N mg) | (mM) | α-$NH_2$ (N mg) | (mM) | α-$NH_2$ (N mg) |
| Taurine | 0·66 | (9·2) | 0·66 | 9·2 | 2·90 | (40·6) | 2·90 | (40·6) |
| Aspartic acid | 0·11 | 1·5 | 0·23 | 3·2 | 0·15 | 2·1 | 1·10 | 15·4 |
| Threonine | 0·34 | 4·8 | 0·34 | 4·8 | 0·22 | 3·1 | 0·64 | 9·0 |
| Serine | 0·48 | 6·7 | 0·45 | 6·3 | 0·17 | 2·4 | 0·60 | 8·4 |
| Asparagine + glutamine | 0·97 | | 0·00 | | 1·00 | | 0·00 | |
| Glutamic acid | 0·16 | 2·2 | 1·00 | 14·0 | 0·27 | 3·8 | 1·26 | 17·6 |
| Proline | 1·08 | 15·1 | 1·00 | 14·0 | 0·13 | 1·8 | 0·27 | 3·8 |
| Glycine | 3·30 | 46·2 | 3·40 | 47·6 | 0·55 | 7·7 | 1·56 | 21·8 |
| Alanine | 0·84 | 11·8 | 0·80 | 11·2 | 0·78 | 10·9 | 1·44 | 20·2 |
| Valine | 0·16 | 2·2 | 0·17 | 2·4 | 0·16 | 2·2 | 0·24 | 3·4 |
| Cystine | Trace | | L | L | 0·10 | 2·8 | L | L |
| Methionine | 0·05 | 0·7 | L | L | 0·07 | 1·0 | L | L |
| Isoleucine | 0·08 | 1·1 | 0·07 | 1·0 | 0·11 | 1·5 | 0·42 | 5·9 |
| Leucine | 0·23 | 3·2 | 0·12 | 1·7 | 0·32 | 4·5 | 0·70 | 9·8 |
| Tyrosine | 0·05 | 0·7 | 0·05 | 0·7 | 0·14 | 2·0 | 0·16 | 2·2 |
| Phenylalanine | 0·05 | 0·7 | 0·05 | 0·7 | 0·14 | 2·0 | 0·20 | 2·8 |
| Lysine | 0·27 | 3·8 | 0·28 | 3·9 | 0·54 | 7·6 | 0·60 | 8·4 |
| Histidine | 0·15 | 2·1 | 0·14 | 2·0 | 0·00 | 0·12 | 0·12 | 1·7 |
| Arginine | 2·86 | 40·0 | 2·80 | 39·2 | 0·85 | 11·9 | 0·91 | 12·7 |
| Glycerophospho-ethanolamine | | | | | 0·19 | | 0·00 | |
| Phosphoethanol-amine | | | | | 0·35 | | 0·00 | |
| Ethanolamine | | | | | 0·00 | | 0·30 | |
| Total (excluding taurine*) | | 142·8 | | | | 67·3 | | 143·1 |

* Does not contribute to α-amino N as determined by Pope & Stevens' (1939) method.
L = determination lost.

extract), 12·8 per cent amino acid N (other than α-amino N), 10·5 per cent taurine N, 3·8 per cent amide N, 3·5 per cent volatile base N and 3·2 per cent N in G.P.E.†, P.E.‡ and trimethylamine oxide.

It will be observed that there is a discrepancy in the hepatopancreas figures for α-amino N determined (i) by the method of Pope & Stevens (1939) (123 mg/100 g wet wt.—Table 1) and (ii) by summation of the α-amino N in the amino acids

† G.P.E. = glycerylphosphorylethanolamine.
‡ P.E. = phosphorylethanolamine.

determined by the Moore & Stein technique (67·3 mg/100 g wet wt.—Table 2). However, hydrolysis of the hepatopancreas extract increased the concentrations of all amino acids (Table 2), showing that peptides soluble in trichloroacetic acid are present in the hepatopancreas. The Pope & Stevens' method depends on the formation of soluble copper salts of amino acids, the copper then being estimated iodometrically. It is known that small peptides also form soluble copper salts (Scaife, 1959), and therefore would contribute to the $\alpha$-amino N determined by Pope & Stevens' method. Thus the higher value obtained by the copper method is probably due to the presence of peptides in the extract.

## DISCUSSION

The values for amino acid concentrations in muscle reported here show a fair measure of agreement with those of Camien *et al.* (1951), although individual variations are to be expected. The latter workers used microbiological methods and determined the amino acid concentrations in acid hydrolysates of muscle extracts. The main differences between the two analyses are in the concentrations of aspartic and glutamic acids, and in the concentration of arginine. With regard to the first two, Florkin (1960) has stated that these two amino acids occur largely as their amides, which is consistent with my findings. In the same article Florkin also reports that the arginine value of Camien *et al.* (1951) was in error by a factor of ten. Their value for the concentration of this amino acid in muscle now stands at 945 mg/100 g wet muscle compared with 497 mg reported here.

The results in Table 2 show that no peptides are present in the abdominal muscle of *Astacus pallipes*. After acid hydrolysis only aspartic and glutamic acid increased in concentration, and this increase was wholly due to conversion of the respective acid amides. Likewise, Shaw (1958) found no evidence of any peptides in the muscle of *Carcinus maenas*. Crustacean muscle seems to contrast sharply with vertebrate muscle in which several dipeptides containing $\beta$-alanine, e.g. anserine and carnosine, are commonly found.

In comparison with the related marine decapod *Homarus vulgaris*, the tissues of which were examined by Kermack *et al.* (1955), the most interesting feature of *Astacus pallipes* is perhaps the absence of betaine from the tissues. This substance makes up 12 per cent of the non-protein nitrogen in *Homarus vulgaris* muscle and 14 per cent in the hepatopancreas.

All the other nitrogenous substances found in *Homarus vulgaris* were also present in *Astacus pallipes* but generally in much lower concentration. Kermack *et al.* (1955) found 749–820 mg non-protein nitrogen in *Homarus vulgaris* muscle and 625 mg non-protein nitrogen in the hepatopancreas. The values for *Astacus pallipes* are 411 and 380 mg non-protein nitrogen. This lower concentration is considered (Camien *et al.*, 1951) to be correlated with the low osmotic pressure of the environment of *Astacus pallipes*.

Of the free amino acids present in lobster muscle Kermack *et al.* (1955) found that quantitatively glycine and proline followed by taurine were the most important. The same substances together with arginine are quantitatively the most important



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in the muscle of the crayfish. It can be inferred from the results of Kermack *et al.* that there is less arginine in lobster muscle (ca. 1·3 mM/100 g) than I have found in crayfish muscle. N-phosphorylarginine is the crustacean phosphagen, and one would expect relatively large amounts of arginine to be present in crustacean muscle generally.

In the hepatopancreas of *Homarus vulgaris*, Kermack *et al.* (1955) found high concentrations of free glycine and taurine. Taurine was the most abundant free amino acid in the hepatopancreas of *Astacus pallipes* and there were also relatively large amounts of free arginine, glycine, alanine and lysine. Histidine was only detected after hydrolysis of the hepatopancreas extract.

It has already been pointed out that peptides were present in the hepatopancreas of *Astacus pallipes*. These were revealed colorimetrically in the effluent fractions of a Moore & Stein column; the most conspicuous of them occurred (i) shortly after the emergence of taurine, (ii) immediately after valine, and (iii) immediately after leucine. No attempt was made to determine the composition of these peptides.



It may be that these peptides are autolytic products of the proteolytic enzymes said to be present in the hepatopancreas of *Astacus pallipes*. Some short time elapsed between the isolation of the tissues and the inactivation of the enzymes by a protein precipitant. It was not possible to follow the desirable practice of freezing the tissues in liquid oxygen immediately after their isolation. The presence of peptides in the hepatopancreas is indicative of the metabolic importance of this organ in Crustacea for even if they are autolytic artifacts they show that the proteolytic enzymes in the hepatopancreas are very powerful.

The Moore & Stein analysis revealed for the first time the presence of G.P.E. and P.E. in the hepatopancreas of *Astacus pallipes*. Since this may not be considered unequivocal evidence of their presence, further evidence was obtained by applying the methods of Campbell & Work (1952), who were among the first to identify these substances in vertebrate tissues.

By chromatographing a concentrated aqueous extract of hepatopancreas on a column of zeo-carb 225 (previously activated with 5 N-HCl) three acidic ninhydrin-positive substances were separated from the remainder. From the Moore & Stein analysis and by comparison with the results of Campbell & Work (1952) these were probably G.P.E., P.E. and taurine.

Paper partition chromatography confirmed that the latter two were in fact P.E. and taurine. In no solvent could they be separated from authentic samples of P.E. and taurine. No authentic sample of G.P.E. was available. This substance was therefore separated from taurine and P.E. by chromatography on a paper column as described by Campbell & Work. It was then found that:

(i) acid hydrolysis resulted in the formation of ethanolamine;
(ii) phosphorus was present in the substance and the most probable N:P atomic ratio was 1:1;
(iii) hydrolysis with 0·5 N-barium hydroxide resulted in the formation of $\alpha$ and $\beta$ glycerophosphate.

These results together with the results of the Moore & Stein analysis may be considered satisfactory evidence for the presence of G.P.E. and of P.E. in the hepatopancreas of *Astacus pallipes*.

G.P.E. and P.E. have been found in mammalian tissues; they occur in highest concentration in metabolically important sites (liver, pancreas) (Tallan *et al.*, 1954). They are thought to be intermediates in the metabolism of phosphatidylethanolamine and current evidence is that they are intermediates in the synthesis of this substance (Kennedy & Weiss, 1956). As with peptides, they may have appeared in *Astacus pallipes* hepatopancreas as a result of autolysis. Nevertheless their presence may again be taken as evidence of the importance of the hepatopancreas in crustacean intermediary metabolism.

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# CARDIOREGULATION IN AN ANEURAL HEART*

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**Abstract**—1. The aneural branchial heart of the California hagfish (*Eptatretus stoutii*) was used in these experiments. The evidence indicates that the myocardium of this species possesses a pressure-sensitive, diffuse, myocardial pacemaker.

2. Electron microscopic studies have revealed in the heart of this species heretofore undescribed atypical subendothelial cells which strongly resemble secretory cells.

3. Biochemical studies, in extension of earlier work, indicate that pacemaker activity is linked to a small-molecule metabolite found in the heart (but not in the liver). This cardioaccelerator substance is not species specific, but it will exert its effect on the hearts of frog and mussel as well as on that of the hagfish.

4. It is suggested that there may be a relationship between the atypical cells and the cardioaccelerator material.

5. It is concluded that cardioregulation in the aneural branchial heart of the hagfish is accomplished by two major factors: tension as governed by venous return and a biochemical factor produced in the heart.

## INTRODUCTION

CARDIOREGULATION presupposes that the normal myocardium is able to adapt itself to the circulatory demands imposed upon it by the organism. Two factors are of prime importance in this regard: first, the stroke volume; and, second, the rate at which the heart beats.

The stroke volume, or volume of blood expelled from the heart per contraction, is dependent upon the venous return to the heart. Consequently, and within definite limits, an increased diastolic filling precedes a greater stroke volume on the part of the myocardium. This general statement is usually referred to as "Starling's law of the heart" (1918). Actually, however, the foundation for such a concept was laid some years earlier by the work of Frank (1895).

The heart rate at a given moment is the resultant of many factors impinging upon the myocardium. Among these are temperature, nervous influences from higher centers (accelerator and/or depressor), inherent rhythmicity, endogenous metabolism and humoral influences imparted by the blood itself. Another condition of extreme importance with regard to heart rate (as well as to stroke volume) is the degree of stretch to which the cardiac muscle is subjected during diastole, whether from internal or external sources. The relevance of this latter factor to the present study is such that it will be considered in some detail.

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Using the neurogenic adult *Limulus* heart Carlson (1904a,b) found an augmentation of the rate when external tension was applied to this organ. Even more pronounced acceleration was found when the filling pressure was increased by introduction of fluid into the lumen of the heart (Carlson, 1907). However, if the ganglion was removed, such an acceleration failed to occur in either case. Unfortunately, he did not report similar experiments on the non-innervated myogenic *Limulus* embryo heart (Carlson & Meek, 1908). Carlson also reported experiments dealing with the effects of tension on the heart rates of various crustaceans and molluscs (1904b, 1906), notably studies using the myocardium of the giant California slug *Ariolimax*. In addition to extending his *Limulus* studies to this species he found that increased intraventricular pressure actually induced a beat in fatigued hearts which again ceased when the perfusion fluid was withdrawn.

Recently Blinks (1956) working with the dog and rabbit, Pathak using the frog, rabbit, dog, rat and guinea pig (1957, 1958a,b) and Keatinge on the rabbit, tortoise and frog (1959a,b) independently found that an increase in heart rate could be elicited by increased external tension and/or internal filling pressure.

The foregoing studies indicate clearly that myocardial distension, especially of the right atrium, is effective *per se*, in producing cardioacceleration in a wide variety of species. Based on his studies using the neurogenic *Limulus* heart, Carlson suggested (1907) that tension would mediate its acceleratory effect in the heart (regardless of species) via stimulation of intracardiac nerves, rather than by direct action on the muscle, a view still held today.

A critical test of this hypothesis would rest upon experiments performed on an aneural heart.

Almost 60 years ago Greene discovered that the branchial (or systemic) heart of the adult California hagfish is aneural (1902a,b). Subsequent work has confirmed this observation (Carlson, 1904c; Fänge & Östlund, 1954; Augustinsson *et al.*, 1956; Jensen, 1958; Results section 4). In the conclusion to his paper on the hagfish, Greene stated: "Any regulation of the heart's action must depend upon the conditions which affect the muscle directly, i.e., tension, nutrition, etc. The volume and pressure of the blood coming to the heart and the changes in the pressure upon the internal organs produced by the ever-varying movements of the plastic body of the animal are the factors that must have a decided influence on the hagfish heart."

Throughout the course of earlier studies by the writer (Jensen, 1957, 1958) pertaining to certain physiological and biochemical aspects of the hagfish heart, as well as others to be reported here, it was repeatedly observed that the isolated hearts accelerated for several minutes following the mechanical disturbance attendant upon lavage of the chamber with medium of the same composition and temperature. It was also noted that manipulation of the heart during the dissection sometimes accelerated the beat. Investigation of these casual observations followed under more controlled experimental conditions. The data to be presented indicate that increase in the rate of the branchial heart of the hagfish is in part dependent upon the diastolic volume: hence one factor governing cardioacceleration in this

non-innervated but spontaneously contractile organ is the action of a pressure-sensitive myocardial pacemaker. Data are also presented which suggest that this aspect of cardioregulation is related to the operation of a biochemical mechanism.

## MATERIALS AND METHODS

Two species of animals were employed for this investigation: California hagfish, *Eptatretus stoutii* (= *Polistotrema stoutii*) and grassfrogs (*Rana pipiens*).

The hagfish were collected by the method and at the locality previously described (Jensen, 1959). Mature specimens of both sexes were employed; no differences were noted in their cardiac activity. After collection, the animals were kept at about 10°C in well-aerated sea water which was changed frequently. It was unnecessary to feed them, as they remain thus in excellent condition for over 5 months in the laboratory; however, the experiments here reported were conducted within 2–3 months after collecting the animals. Specimens collected throughout the year showed no evident seasonal differences in their myocardial activity.

The grassfrogs came from a commercial dealer in Oshkosh, Wisconsin. All males were specially selected for pregnancy tests. (Average length: 7·4 cm nose to crotch; weight: 31 g.) They too were kept at 10°C, but were used within a week or so after arrival.

The hagfish hearts were prepared for recording as follows: the animal was grasped by the head with a hemostat, and the brain crushed. Another hemostat was clamped on the caudal fin and the fish stretched out on a board. A midline incision revealed the liver: the anterior lobe was deflected, exposing the branchial heart and ventral aorta. A thread was placed about the aorta and later attached to the strain gage for recording. Another thread, containing a small loop, was then securely tied about the atrioventricular junction or the cardinal veins to secure the heart to a glass hook in a constant temperature chamber. Generally, only the ventricle was used. Never did the atrioventricular ligature induce an interruption of the ventricular rhythm.

The frogs were pithed, the heart exposed, and a thread sutured into the ventricular apex. A second thread with the small loop described above was secured about the aortic trunks near their bifurcation just anterior to the truncus arteriosus. Thus, atrial and ventricular contractions were both recorded.

After placement of the threads, the hearts were rapidly excised and placed in the constant temperature chamber controlled to within ± 0·1°C and calibrated to 10·0 ml. Artificial sea water (Jensen, 1958) was used for the hagfish studies (pH 5·8) and Ringer's solution (Prosser, 1952) without glucose (pH 7·2) for the frog hearts. All media, extracts, etc. were made with glass-distilled water. The media were gassed continuously with 95% $O_2$+5% $CO_2$ in all studies, and the temperature maintained at 15°C, except where otherwise noted (Table 1). Under these conditions, the frog hearts could be maintained routinely *in vitro* at a steady spontaneous rhythm without internal perfusion for 4 hr or longer, whereas the hagfish preparation would beat for over 18 hr. Before the start of any experiment, the heart was allowed to "equilibrate" in the apparatus for at least half an hour; if spontaneous irregularities in the beat occurred during this interval, the heart was discarded.

Before the addition to the chamber of any solution, its pH and temperature were carefully adjusted, and a volume of fluid equivalent to that being added was removed so that the final volume in all cases was 10·0 ml. When the additives were washed out of the chamber, a minimum of 100 ml of appropriate medium at the same temperature was used to effect this lavage.

Isotonic contractions were recorded by placing a weak spring in series with the thread from the heart to a Model G7A Statham transducer. After suitable amplification (Brush universal D.C. amplifier) the tension developed by each contraction was recorded on one channel of a Brush two-channel oscillograph. Silver–silver chloride electrodes contained in small glass tubes filled with saline solution were placed directly on each side of the ventricular base to record the spontaneous action potentials (electrocardiographs). This position was chosen arbitrarily as it interfered least with the movement of either heart or electrodes during systole, and permitted reasonably consistent results from different preparations. The actual configuration of the potentials would, however, vary somewhat depending upon the exact placement of the electrodes on the epicardial surface. These potentials were amplified (Brush high gain D.C. amplifier) and recorded simultaneously with the myogram on the adjacent channel of the oscillograph. This latter instrument was equipped with a timing device to give signals at 1 sec intervals which were recorded at the bottom of the chart at the same time as the other data. The oscillograph was equipped with six speeds, enabling continuous recording during a given experiment at chart rates up to 12·5 cm/sec. Thus, action potential, tension developed and heart rate could be recorded and the individual cardiac cycles expanded or compressed on the graph in time as required for a given study.

Each preparation served as its own control; where possible, several observations were made on the same heart. No attempt is made to report absolute values, but rather comparative figures relating control and experimental findings in the same preparation. However, a number of similar experiments were conducted on different heart preparations; the exact number is summarized under Results.

For the distension studies, a cannula was constructed from the shank of a 27 gage hypodermic needle connected to a 0·25 ml tuberculin syringe by a fine polyethylene catheter (i.d., 0·015 in. × o.d., 0·043 in.). The needle was inserted into the lumen of the hagfish ventricle through the aorta, and its tip gently forced past the ventriculoaortic valve during systole so that it was placed in the ventricular lumen. In the frog, the cannula was inserted directly into the ventricular cavity through the apex and secured in place by a tight mass ligature which also served, in both hagfish and frog, to connect the heart with the strain gage.

A Grass stimulator was used where indicated, and the artificial stimuli were delivered through silver–silver chloride electrodes in direct contact with the epicardial surface.

For routine histological examination, the hearts were fixed by perfusion with Bouin's fluid and stained with hematoxylin and eosin after paraffin imbedding and sectioning at 6 μ. Ventricles for electron microscope study were cut at approximately 0·05 μ or less after osmium fixation. No special staining techniques were employed. An RCA electron microscope was used for examination and photography.

The tissue extracts were prepared as follows: After collection of a large number of hagfish, the branchial hearts were dissected from the animals and freed of excess fat and connective tissue. After blotting on Whatman No. 1 filter paper, the still-beating hearts were frozen immediately and pooled in a jar chilled by a dry ice–acetone bath. They remained frozen until subjected to lyophilization, usually within 2 or 3 days. The dried tissue was then thoroughly ground in a mortar, and the coarse reddish brown hagfish heart powder stored in tightly sealed jars at −5°C until needed for extraction. Ten lots of this material were thus prepared, representing the myocardia from over 4000 fish. The powder retained its biological activity even when stored for periods of up to 1 year before extraction.

Hagfish liver powder and rabbit heart powder were similarly prepared for extraction from the respective tissues after thorough exsanguination.

Hagfish heart extract (HHE) was prepared by placing a weighed quantity of the powder in a mortar and thoroughly triturating with a known volume of appropriate solvent. This was usually the medium in which the succeeding experiment was to be conducted, or

glass-distilled water if further manipulations were to be carried out on the extract. (In general, 0·1 g of powder was treated with 1·0 ml of solvent.) The system was then subjected to centrifugation at 5000 rev/min for 20 min. Following such treatment, the material was separated into three sharply-defined layers; an upper fatty zone, a clear reddish middle area and a firmly-packed pellet of tissue-detritus at the bottom. The clear middle layer was carefully removed to the exclusion of the other areas, and is termed HHE. Hagfish liver and rabbit heart extracts were similarly prepared.

Further experimental treatment of the HHE is dealt with fully where appropriate (see Results).

Hematocrits were obtained by taking blood samples in capillary tubes from the hagfish ventral aorta immediately anterior to the heart, the tubes were sealed at one end and centrifuged for 15 min at 5000 rev/min.

The following drugs were used in this study: α-epinephrine-bi-tartrate from the California Corporation for Biochemical Research, Los Angeles, 63; Isuprel hydrochloride (Isoproterenol hydrochloride) from the Winthrop Laboratories, New York 18, N.Y.

## RESULTS

### 1. *Distension studies*

Large adult hagfish of similar size were selected for these experiments so that the results might be comparable. The average length of the animals was 39 cm

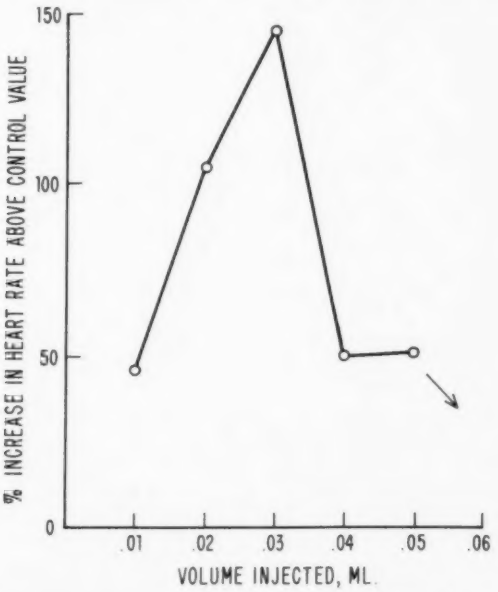


FIG. 1. Each point represents the average value of five observations. Ten ventricular preparations were used. Arrow at 0·05 ml on abscissa indicates that neither electrocardiographs nor myograms were detectable at higher injection volumes.

and the average weight 72 g. The isolated, spontaneously beating ventricle was selected for cannulation as it was possible to obtain preparations without leaks under controlled conditions in which known volumes of saline solution could be

introduced and withdrawn at will. In all, seventy-one observations of this sort were carried out, using different volumes of fluid. A total of twenty-seven hagfish ventricular preparations were included in this group of experiments. Of these, twenty-three (85 per cent) responded to an increase in intraventricular volume by an increase in rate, not force, of the beat. Four ventricles failed completely to alter their rhythm for no accountable reason.

Fig. 1 indicates the increase in rate above the control level plotted against the volume of saline injected. Although the rates of the individual hearts varied widely from experiment to experiment and in the same preparation during the

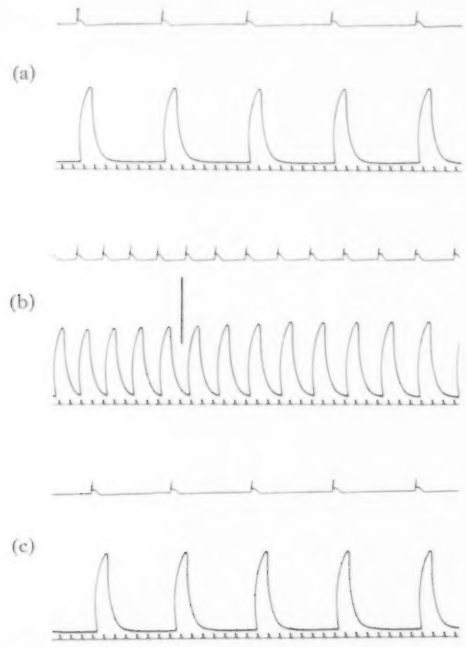


FIG. 2. Effect of mechanical distension on the isolated spontaneously beating hagfish ventricle. (a) Control record, rate = 8 beats/min. (b) Rate at approximately 30 sec after injecting 0·03 ml of saline = 30 beats/min. At mark, fluid was withdrawn. Note gradual slowing and slight positive force increase. (c) 3 min after (b). Rate = 8 beats/min.

In all figures, the upper trace of each chart segment indicates the spontaneous action potential, the middle record is the myogram, and the bottom record is the time signal at 1 sec intervals.

course of several studies, it may be seen from the graph that, over a narrow volume range in hearts from fish in the size range indicated, the percent change in rate is approximately proportional to the volume injected. The response to distension occurred almost immediately; it was sustained until the excess fluid was withdrawn, at which point the rate gradually returned to its control value. This usually occurred in about 2–3 min and was a consistent finding (see Fig. 2).

It was repeatedly noted that the introduction of volumes of about 0·04 ml or greater caused the rate decrease recorded in Fig. 1 except when hearts from larger fish (about 50 cm in length) were used. In addition, a consistent decrease in the force of the beat was observed upon ventricular distension regardless of volume injected in these studies.

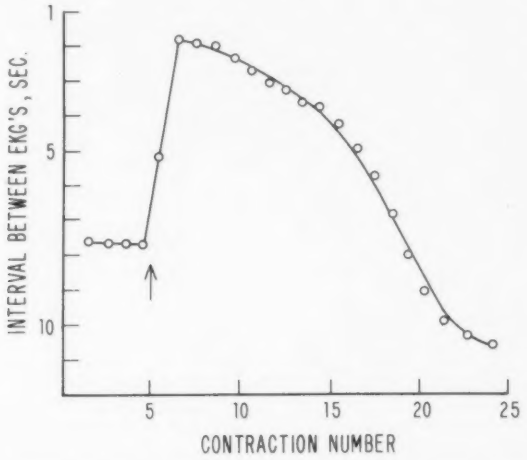


FIG. 3. Isolated spontaneously beating hagfish ventricle with small puncture to permit fluid to escape. At arrow, 0·01 ml of saline was injected. Total elapsed time from contraction 1 to 24 was 132 sec.

The phenomenon of cardioacceleration produced by stretching the myocardium was by no means limited to the ventricular cannulation experiments. For example, injection of 1·5 ml of saline into the portal vein of several large specimens *in situ* induced a rate change in the systemic heart from about 34 to 44 per minute. In addition, a slight tension induced by stretching the isolated heart between pairs of forceps caused a rate increase as did manipulation of the thread and aspiration of a small bit of epicardium into the tip of the electrodes during their adjustment *in vitro*. Another method found successful in producing cardioacceleration *in situ* was "milking" the blood toward the heart from the posterior cardinal and portal veins by gently stroking these vessels simultaneously in a posteroanterior direction with a finger.

In six preparations, the ventricular epicardia were punctured to impart a very slight leak to the system; thus, a small quantity of medium could be introduced during diastole which would be expelled during the succeeding systoles. By pulsatile injections of 0·01 or 0·02 ml of saline during the diastolic interval, it was found possible approximately to double the control rate for as long as the injections were continued, deceleration occurring as the perfusion was stopped. The tension had to be maintained for several seconds, otherwise acceleration would not occur. A single very rapid stretch produced no effect on the rate.

As controls, two spontaneously beating frog hearts were cannulated (*vide supra*) and Ringer's solution introduced at volumes ranging from 0·01 to 0·08 ml in steps of 0·01 or 0·02 ml. Absolutely no rate effect was obtained in twelve trials; however, a clear-cut and definite increase in force of the contractions was obtained repeatedly in both preparations. Within limits, this force increase was related to the volume of fluid introduced.

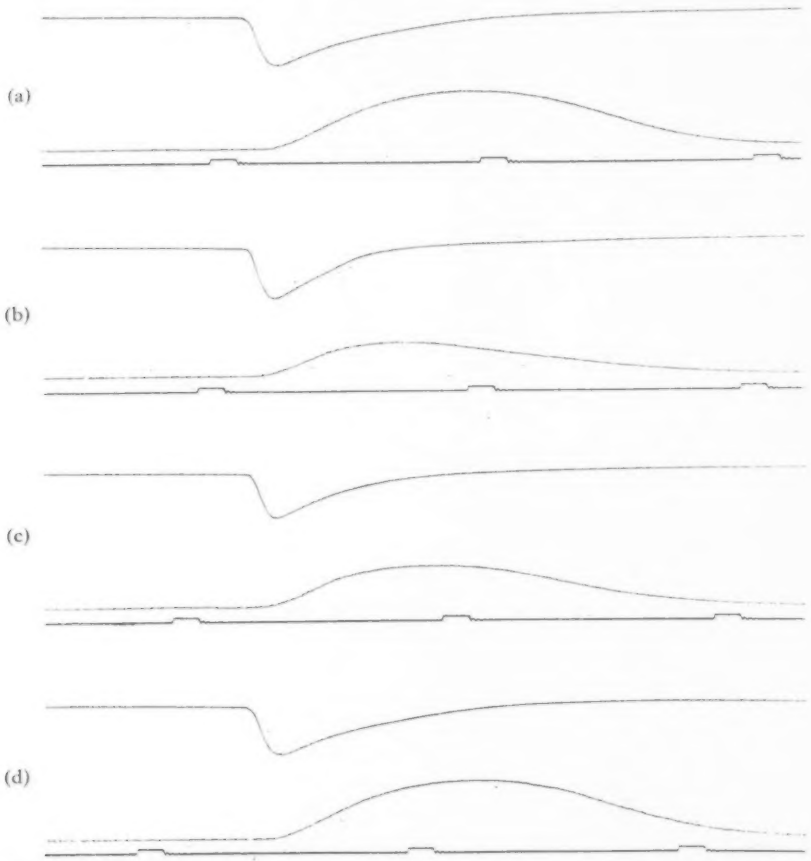


FIG. 4. High-speed records of several contractions of a spontaneously beating hagfish ventricle (punctured) before and after mechanical stretch. (a) Control contraction. (b) Contraction immediately following introduction of 0·03 ml of saline. (c) Fifth contraction after introduction of saline. (d) Tenth contraction after introduction of saline. Note the decreased force of the contraction in (b) and (c).

Fig. 3 illustrates a time-curve of acceleration and deceleration during and after distension of a hagfish ventricle which, as previously described, had a small puncture in the epicardium to permit the fluid to escape. As the rate during this experiment was changing, the time interval between the start of successive EKG's

is plotted on the ordinate, and this figure is placed at the midpoint of successive contractions on the abscissa. This curve was obtained from one of three similar experiments performed on this ventricle; it illustrates the rapid acceleration followed by the gradual decline in rate as the fluid is expelled. The final rate was somewhat lower than the control value. This figure qualitatively rather than quantitatively exemplifies a fact observed three times in one heart and also in several other preparations.

Fig. 4 shows a series of contractions obtained when the oscillograph was running at 12·5 cm/sec in order to study in more detail the individual cardiac cycles. Taking the sharp downstroke on the EKG as the depolarization phase, this was markedly accelerated immediately following the introduction of 0·03 ml of saline. The average time for this interval derived from several control observations was 104 msec, while the comparable figure for the three successive contractions after distension was 88 msec, or a decrease of about 15 per cent. Similarly, the latent period of systole was reduced from 82 to 72 msec for the same contractions (−12 per cent). The contraction phase declined about 11 per cent, whereas the relaxation phase remained about the same. It is also worthy of remark that once again a decrease in force of the contraction was observed.



2. *The effect of temperature on the rate of the branchial heart*

The results in this section are summarized in Table 1. The average rates of the systemic heart in thirty fish of both sexes were found to be 16/min at 13°C and 30/min at 26°C. Thus, the rate approximately doubles for this 13° rise in temperature. Studies *in vitro* indicated that the entire branchial heart beats at a somewhat faster rate than the isolated ventricle alone. It is interesting to note that the branchial heart beats at a somewhat faster rate when first isolated than *in situ*. This probably reflects in part mechanical effects resulting from setting up the preparation as well as the slight external tension under which the organ is placed for recording. A number of observations on the rate of the spontaneously beating isolated frog heart are included in Table 1 for comparative purposes. Generally the heart of this species beats at a much higher rate at 15°C than did the branchial heart of the hagfish.

In a series of hagfish heart observations, the heater in the water bath was shut off while the refrigeration unit continued to run, allowing the temperature in the chamber to descend gradually toward 0°C. At about 1°C the rate was much reduced (about 3–4 beats/min), but usually the contractions remained strong and regular. Conversely, as the temperature was permitted to approach or exceed 30°C, the beat generally weakened and became erratic. Consequently, it appears that the myocardial function of these animals is in part dependent upon rather low temperatures, a fact borne out by the observation that the hagfish will not survive long in aquaria at temperatures substantially exceeding 20°C. This also is of interest in relation to the general habitat of these Cyclostomes since in Southern California they occur in much deeper waters than in the Northern latitudes, although the temperatures in the two areas are approximately the same.

TABLE 1—EFFECT OF TEMPERATURE ON THE RATE OF THE HAGFISH BRANCHIAL HEART

| Group | Species | Sex | Preparation* | No. of animals | Temp. (°C) | Heart rate† (beats/min) |
|---|---|---|---|---|---|---|
| 1 | *E. stoutii* | Mixed | Branchial heart *in situ* | 30<br>30 | 13<br>26 | 16 ± 0·73<br>30 ± 1·85 |
| 2 | *E. stoutii* | ♂ | Branchial heart *in situ* | 18<br>18 | 13<br>26 | 15 ± 0·886<br>30 ± 2·10 |
| 3 | *E. stoutii* | ♀ | Branchial heart *in situ* | 12<br>12 | 13<br>26 | 18 ± 3·37<br>31 ± 2·16 |
| 4 | *E. stoutii* | Mixed | Isolated branchial heart | 20 | 15 | 26 ± 1·35 |
| | | | Isolated ventricle of branchial heart | 20 | 15 | 16 ± 0·684 |
| 5 | *R. pipiens* | ♂ | Isolated heart | 33 | 15 | 37 ± 1·13 |

* Groups 1, 2 and 3: the fish were pinned to a board and a small ventrolateral incision made on the left side revealing the branchial heart: the rate was counted three times for each fish at each temperature by three independent observers. These closely agreeing rates were averaged, and used in the Table. Groups 2 and 3 represent Group 1 separated according to sex. There is no significant difference between the rates of each sex at either temperature (*t*-Test). The rates for Groups 4 and 5 were obtained directly from the oscillograph record about 10 min after the preparation was set up.

† Including standard error of the mean.

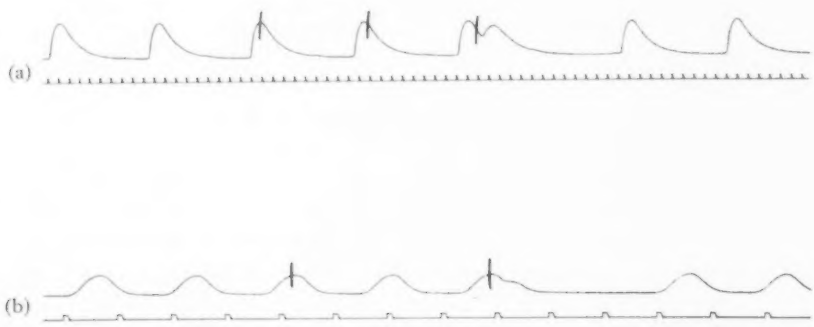


FIG. 5. (a) Determination of refractory period of hagfish ventricle. Single square wave pulses (150 V, 2 msec duration) were delivered at points indicated by vertical bars on myograms. No electrocardiographs were recorded. (b) Frog ventricle for comparative purposes. Stimuli applied as above. Note that in (b) the recording chart speed was somewhat faster. Time signal, 1 sec in both cases.

3. *Electrical stimulation of the hagfish ventricle*

Five isolated hagfish ventricles were employed for these studies; two frog hearts served as controls. Fig. 5(a) illustrates the fact that in no case could an extrasystole be induced until the heart was well into diastole, indicating that the refractory period in these organs is quite long. This observation was repeatedly confirmed in all preparations, and usually the heart failed to respond until much

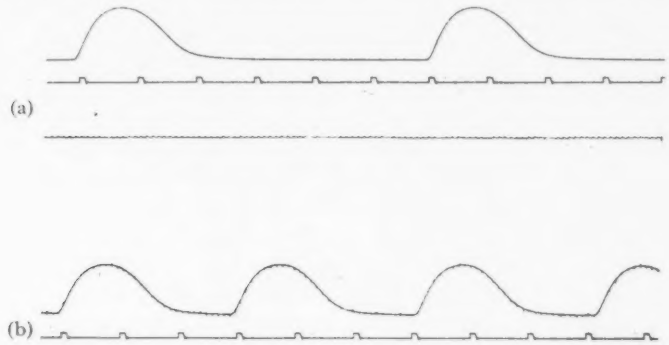


FIG. 6. Effect of artificial stimulation on the rate of a spontaneously beating hagfish ventricle. (a) Control rate = 12 beats/min. Signal magnet trace omitted from top of this chart. (b) Stimulated at a rate of 10/sec, at 100 V with 2 msec square wave pulses. Rate during stimulation = 24 beats/min. (Third trace from top is of signal magnet indicating when stimuli were applied.)

further in diastole than illustrated in the figure. (See also Fig. 6.) By contrast, Fig. 4(b) shows that under similar conditions, stimulation of the frog ventricle at about the peak of its contraction resulted in an extrasystole and compensatory pause. This was a consistent finding in the frog heart.

Attempts to induce quiescence in the hagfish ventricle by atrioventricular ligation in order to study the staircase phenomenon failed completely as did low temperature. In a non-beating frog ventricle (Stannius' ligature number 2) the staircase effect could be readily produced. The threshold voltages for inducing ventricular extrasystoles in the hagfish were consistently higher than in the frog. However, a wide range of voltages were tested on each preparation, and levels much higher than threshold were used.

Attempts to stimulate the hagfish ventricle with currents of 100 V (2 msec square wave pulses) applied at rates of 30/min, 10/sec or 50/sec, produced an acceleration of the rate; however, as tested, no stimulus was effective until late in diastole as with the single pulses (see Fig. 6).

4. *Histological studies on the hagfish heart*

The microscopic anatomy of the hagfish ventricle presents a number of unusual features which are of sufficient importance to warrant their inclusion here. Although an earlier paper mentioned the lack of neural elements in the myocardium (Augustinsson *et al.*, 1956), no photographs were published, nor was the histology

of this organ presented in any detail. The following discussion, however, is concerned primarily with those anatomical details which are relevant to a better understanding of the physiological and biochemical data presented in the other sections of Results.

Sections of hagfish branchial hearts stained with hematoxylin and eosin revealed that these organs are invested in a clear-cut pericardium. The epicardial layer was very thin (about three cells thick), and stained very deeply. Under lower magnifications, the myocardium of both auricle and ventricle was extremely diffuse and trabecular in appearance; the structure of the former was far thinner and more delicate-appearing than that of the latter chamber. Study of these slides under an oil immersion lens disclosed typically striated myofibrils, fusiform in shape and imbedded in large quantities of sarcoplasm. There was no evidence of blood vessels to denote a coronary system; however, the general paucity of muscle fibers and the extremely spongy character of the myocardial elements indicate that nourishment of the heart may be readily accomplished by diffusion from the venous blood passing through the chambers. Collagenous connective tissue was quite sparse. The endothelial cells possessed relatively large elongated nuclei; their cytoplasm was flattened and closely invested the strands of muscle fibers, which possessed few nuclei.

Electron microscope studies demonstrated several other interesting details of the fine structure of the hagfish ventricle. Fig. 7 illustrates a longitudinal section of this organ. At the left is a thin sheet of endothelial cytoplasm. Note the irregular size and relative scarcity of the myofibrillae and the numerous mitochondria scattered about in the sarcoplasm. Neither capillaries nor connective tissue are evident. Neural elements were not observed in confirmation of earlier findings. No sarcolemma is present.

Fig. 8 shows an interesting and atypical form of cell which was found scattered throughout the myocardium, always in a subendothelial position, but never found imbedded in the myocardial substance proper. The densely packed, dark-appearing granules were a consistent finding, and the presence of these "secretory" cells taken in conjunction with some of the biochemical data (Results section 5) lend credence to the suggestion that the hagfish heart may possess an endocrine as well as pulsatile function. Subsequent to the discovery with the electron microscope of these atypical structures distributed in random fashion throughout the myocardium, a careful re-examination of the stained sections under an oil immersion lens revealed that they can be distinguished by this means also (but only with great difficulty) from the other myocardial elements. Consequently, it is not surprising that these structures have not been reported heretofore. It is possible, however, that these cells could be revealed more easily by the use of special staining techniques.

5. *Biochemical studies on hagfish heart extracts*

The experiments included in this section represent a number of preliminary investigations dealing with the nature of the cardioactive material reported

FIG. 7. Electron photomicrograph of longitudinal section of hagfish ventricle. Endothelial sheet at left. Large black marks are fixation artifacts. (× 4680).

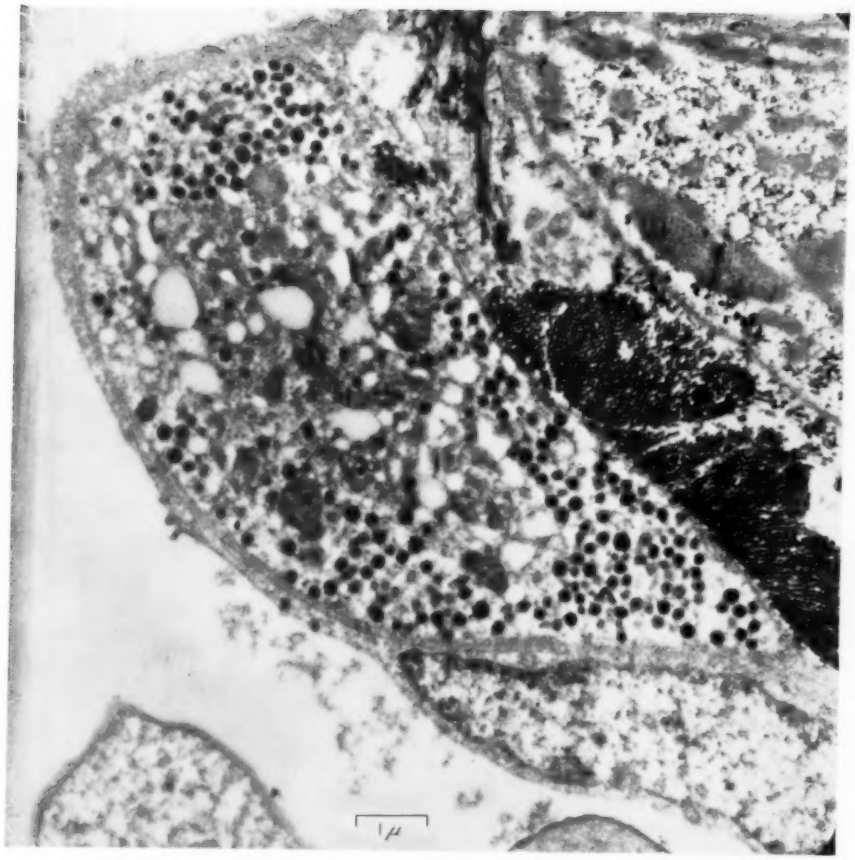


FIG. 8. Electron photomicrograph of hagfish ventricle. At lower right, nucleus of endothelial cell with its thin cytoplasmic layer. In center of field and in a subendothelial location, is an atypical "secretory" cell filled with dark-appearing granules of an unknown nature. The large dark mass above the endothelial cell nucleus on the right is a collagen bundle. Muscle cells may be faintly seen at the upper right. (× 9600).

earlier in crude saline extracts of the systemic heart of the hagfish (Jensen, 1958). The positive chronotropic response of the isolated spontaneously beating frog heart was chosen for bioassay of the extracts, as it was repeatedly observed that

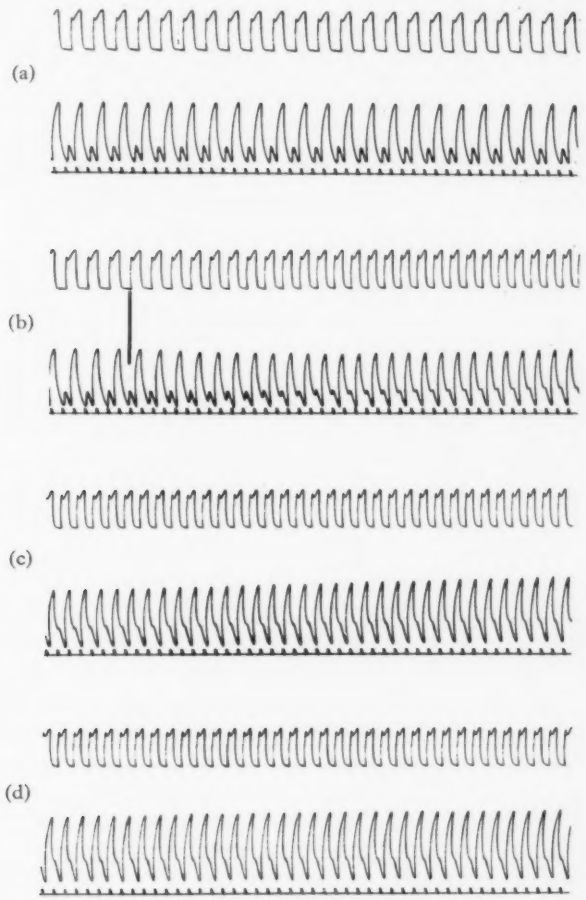


FIG. 9. The effect of HHE on the isolated spontaneously beating heart of the frog. The small peaks on the myogram are auricular and the large peaks ventricular systoles. The action potential was recorded only from the ventricle. (a) Control record. (b) At vertical bar, HHE was added to the chamber. Note progressive rate increase, fusion of atrial and ventricular contraction, and apparent force increase due to this fusion. (c) and (d) are continuations of the record from (b).

these preparations, after a short equilibration period *in vitro*, would accelerate markedly if extracts containing the active material were added to the chamber*.

* This is in distinct contrast to hagfish hearts, which were unresponsive unless isolated for some hours prior to testing. The frog heart, then, affords a distinct advantage for bioassay in this respect.

If, however, the extract was inactivated by preliminary treatment (*vide infra*) the heartbeat rate remained unchanged. Each assay was carried out a minimum of three times, on at least two different hearts.

It should be re-emphasized that in every experiment the pH, temperature, and the volume of the additives were carefully adjusted to that of the Ringer's solution prior to introduction to the test heart, and the final volume was always 10·0 ml.

The experiments here reported were carried out on thirty-one isolated spontaneously beating frog hearts. Every heart served as its own control, and in most instances a number of experiments could be conducted on the same preparation after washing followed by a suitable re-equilibration period.

Fig. 9 illustrates the typical effect of untreated HHE on an isolated frog heart. The control rate was 30 beats/min; 1·5 min after addition of 1·0 ml of HHE (representing the aqueous extract derived from 0·1 g of hagfish heart powder), the rate had accelerated to 44 beats/min and it remained unchanged until the chamber was washed, 26 min after the extract had been added. After washing, the rate gradually returned to the control value in about 10 min. Note that as the rate accelerates, there is a definite fusion of the auricular systole with the ventricular contraction, indicating a decreased atrioventricular conduction time. That this finding is real rather than a recording artifact was revealed by high-speed records which show a definite decrease in the atrioventricular peak to peak distance of the contraction phases. No positive force increase is evident here save that produced by fusion of the systolic phases of both chambers, although such a response was elicited by HHE in several experiments. These findings were confirmed fifteen times on as many preparations using extracts prepared from different lots of hagfish heart powder. It is worthy of comment that acceleration usually took several minutes to reach a maximum, which was then sustained until the chamber was washed; in one study this exceeded 1 hr after the extract was introduced. It is also noteworthy that the return of the rhythm to the control rate was usually quite gradual and occasionally several hundred millilitres of Ringer's solution were required to bring about this reversion. An increasingly greater rate response could be elicited when the concentration of HHE in the chamber was increased by successive additions; however, the data are far from adequate to attempt quantitative treatment at this time.

Neither hagfish liver extracts nor rabbit heart extracts derived from the exsanguinated lyophilized organs in a fashion identical to the preparation of HHE produced the slightest effect on the chronotropism of the frog heart, even though HHE was effective in the same preparations (four each). This despite the fact that both the hagfish liver and rabbit heart extracts represented the soluble material derived from up to five times the mass of tissue (0·5 g) used for the HHE (0·1 g). Aside from the inherent interest of these results, they also serve as tissue extract controls on the other studies.

A quantity of hagfish heart powder of known cardiac activity was ashed in a crucible heated to redness, cooled, taken up in a small volume of Ringer's solution,

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and the pH adjusted with N-HCl. The product was completely inactive in the frog heart.

Earlier reported results on the effects of heating the HHE were inconclusive (Jensen, 1958). Consequently, a quantity of HHE was prepared in Ringer's solution as described in Methods and placed into several heat-resistant 15 ml polyethylene centrifuge tubes: one lot was untreated and served as the control; the remaining batches of HHE were placed in a boiling water bath for 10, 20 and 40 min respectively. After cooling, centrifugation removed the protein coagulum and the clear straw-colored supernate was bioassayed. All fractions save the one heated for 40 min exerted a positive chronotropic effect, indicating that the cardioactive material is relatively thermostable. Here, too, it was noted that the atrioventricular interval decreased. Typical bioassay records were quite similar to Fig. 9.

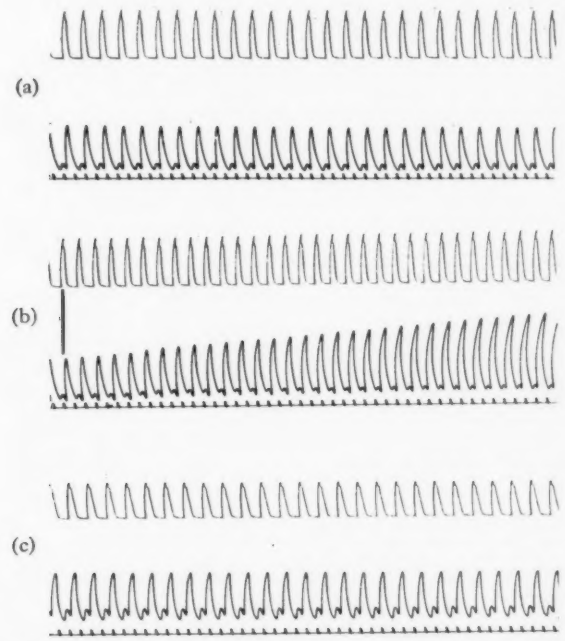


FIG. 10. The effect of α-epinephrine bitartrate ($10^{-4}$ g/ml) on the isolated spontaneously beating frog heart. Compare with Fig. 9. (a) Control rate = 36 beats/min. (b) At mark indicated, epinephrine was added to chamber. Rate in 30 sec = 42 beats/min. Note lack of fusion of atrial and ventricular contractions, marked increase in force of contractions. 6 min after adding the epinephrine, rate = 42 beats/min, but force of beat was markedly less. (c) 10 min after adding the epinephrine, the rate had returned to the control value. The chamber was not washed during this interval.

Another preparation of HHE in distilled water was divided into two portions. The control extract was stored in the cold room (5°C), while the remaining material was dialysed in cellophane tubing against three 3 l. changes of distilled water with

constant stirring at 5°C for 44 hr. After lyophilization and resolution of the residue in Ringer's it was found that the dialysed material was completely inactive, whereas the control extract produced a marked cardioaccelerator effect.

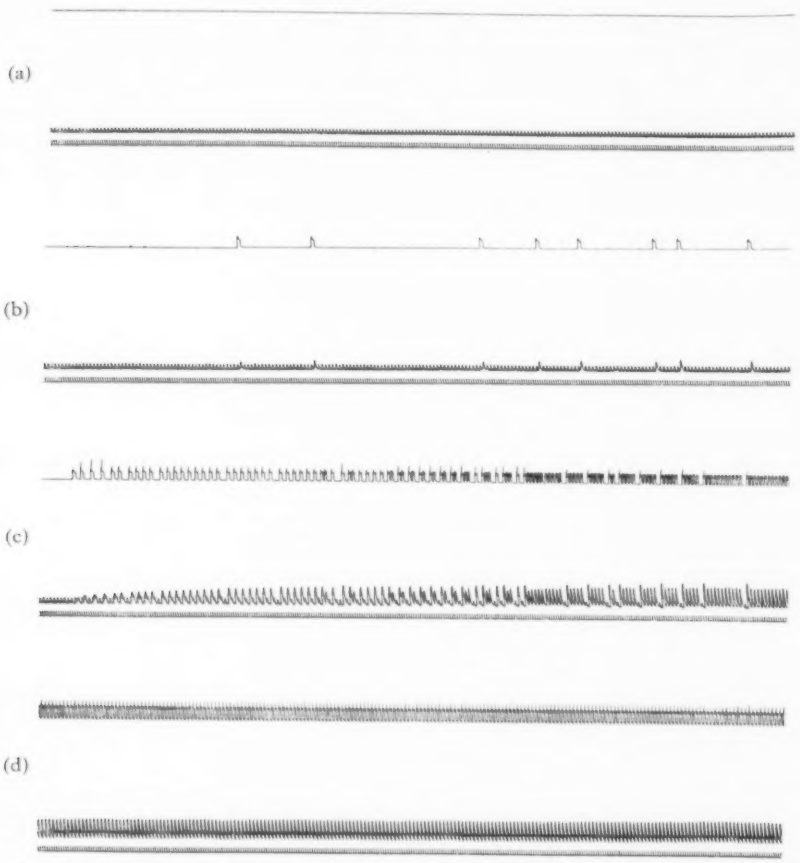


FIG. 11. The effect of HHE on a frog heart with spontaneous total atrioventricular block. (a) Total AV block which had been in effect for 40 min. Note absence of ventricular action potentials. (b) 15 min after placing 1·0 ml HHE in chamber. Occasional ventricular contractions are evident. (c) 5 min after introduction of 1·0 ml additional HHE (20 min from first application of material). Progressively better rhythm is evident from left to right. (d) 1 hr after first application of HHE. The preparation was not washed or otherwise disturbed during this interval.

As the hagfish systemic heart is known to contain a relatively high concentration of catecholamines (Augustinsson *et al.*, 1956), it was conceivable that the effect on the frog heart was due to these compounds. Therefore a series of frog hearts was tested for comparative purposes with various concentrations of α-epinephrine

bitartrate to determine its effect on these preparations under the present experimental conditions. The concentration range employed was $10^{-4}$, $5 \times 10^{-5}$, $10^{-5}$ and $10^{-6}$ g of the salt per millilitre. Fig. 10 shows the rapid response to $10^{-4}$ epinephrine. No atrioventricular fusion occurred. The response to this drug was quite rapid, the maximum ino- and chronotropic effect being reached in about 30 sec and the preparation returned to slightly below its control rate and force spontaneously in 10 min without washing. These findings were consistent at this higher dosage level in four hearts; however, the response to the lower concentration was consistently erratic or non-existent. Briefly, then, the effect of epinephrine in the frog heart was quite irregular in occurrence and evanescent in nature in sharp contrast to the results obtained with HHE on these preparations.

An interesting effect of HHE on the isolated frog heart is recorded in Fig. 11. It was noted in about 60 per cent of the preparations that after repeated treatment with drugs or extracts and subsequent washings, progressive atrioventricular block occurred spontaneously, and finally the ventricle ceased beating entirely except, in some cases, for an occasional contraction; the auricles under these conditions usually maintained an excellent beat. Once this condition developed, it generally did not revert spontaneously; in fact it usually persisted until the run was terminated after several hours. The effect of HHE (from 0·2 g tissue) on this condition is recorded in the figure together with the details of the experiment. Neither epinephrine in the aforementioned doses nor isoproterenol ($0{\cdot}25\times$, $0{\cdot}50\times$ and $0{\cdot}75 \times 10^{-5}$ g/ml) exerted the slightest effect on this heart block save for a slight rate effect on the auricular contractions. As the auricles were beating normally when the HHE was added under these conditions, it is evident that these experiments provide additional evidence that HHE facilitates atrioventricular conduction.

### 6. *Miscellaneous observations*

In this section are included a number of studies, general in nature, which enlarge upon certain aspects of the general cardiovascular physiology of the hagfish.

1. The average blood pH in the ventral aorta (seventeen specimens) was found to be $7{\cdot}70 \pm 0{\cdot}112$ (including standard error of the mean).

2. The hematocrit obtained from aortic blood samples (nineteen fish, mostly duplicate determinations) was $24 \pm 1{\cdot}8$ per cent packed red cells (including standard error of the mean).

3. Average branchial heart weight for ten fish of both sexes (average size: 38·7 cm, 58 g) was 0·146 g or 0·25 per cent of the body weight. This finding is consistent with the earlier report that sluggish fish tend to have a much lower proportion of cardiac tissue to body weight than do more active species (Brown, 1957).

4. During the entire course of this work to date approximately 9000 fish were dissected. Of these, four branchial hearts were found in which the ventricle was dark purple (instead of the usual pink colour), very firm to the touch, and they exhibited not the slightest trace of a pulsation. It was also noted in these specimens that the atria were somewhat dilated and beat normally. Thus, it appears that the systemic ventricle is not essential to survival, since in all other respects these fish appeared to be of normal vigor until this defect was noted upon sacrifice.

5. Three fish were subjected to extirpation of the neurogenic (Greene, 1899) caudal heart by complete ablation of the posterior several centimetres of the caudal fin containing

this organ. 3 weeks later these animals appeared in quite good condition, save for the handicap in swimming occasioned by the loss of the tail-fin. The caudal heart, then, is not an essential organ to survival. The wounds were clean at sacrifice and some healing, albeit slight, was evident.

6. The aforementioned experiments led to another observation of some interest. It was repeatedly noticed, during the routine dissections of the hearts, that clotting of the blood was erratic in its occurrence either *in situ* or otherwise, and when clot formation did occur the resultant mass was very soft and quite friable. When the caudal hearts were removed and the fish returned to sea water for observation, the wounds bled quite freely, and clot formation occurred only on the bottom of the container rather than at the more useful locus in the injured region. It was then observed that the animals secreted masses of slime. This material then became entangled in the wound and bleeding stopped. When the slime was removed, bleeding resumed until the mass of slime was replaced by further secretion. It is therefore suggested that the abundant capacity of these animals to produce this viscous and tenacious material perhaps may serve to protect the fish from excessive hemorrhage.

7. Three hagfish were placed in a bucket containing the medium adopted for the isolated hearts. After remaining for 60 days in this artificial sea water, which was changed weekly or more often, all of the animals were alive and in apparently good condition. At this point, the fish were sacrificed and recordings made of the spontaneous branchial heartbeat. In each of these preparations, the contractions became irregular a short time after isolation, a finding perhaps attributable to the absence of trace elements normally present in natural sea water. This interpretation is even more creditable when one considers that hagfish are normally in ionic balance with sea water (McFarland & Munz, 1958).

8. In five unanesthetized specimens the blood pressure was obtained directly by cannulation of the ventral aorta immediately anterior to the pair of branchial arteries nearest the heart. The average value obtained was 38 mm of blood (range 25–49 mm) in these fish at 20°C. Although these values are rather crude, they are included here to illustrate the low arterial blood pressure found in these fish. This observation is consistent with the fact that the blood merely flows when an artery is severed, and is not ejected in spurts as would occur under a higher pressure.

## DISCUSSION AND CONCLUSIONS

There is now definite evidence for the operation of at least two major factors governing cardioregulation in the aneural branchial heart of the hagfish, factors which Greene (1902a) many years ago suggested would be of importance in this regard. These are: degree of stretch of the myocardium and biochemical (or humoral) influences. A third element, of minor significance to the animal considering the relative stability of its natural habitat, is the effect of temperature on the myocardium.

It has been demonstrated in several ways, both *in situ* and *in vitro* that either the entire hagfish heart or the ventricle alone will accelerate when distended slightly, hence it obviously possesses a pressure-sensitive pacemaker. This heart has given no indication of contracting with greater force when the diastolic filling is increased, as might have been expected on the basis of Frank's (1895) and Starling's (1918) work. On the contrary, as the intraventricular volume is increased. the force of the contraction was found actually to decrease, and this was a consistent observation.

Since the heart of the adult hagfish is aneural, the accelerator mechanism undoubtedly resides within the myocardium. This is not to imply, however, that an empty hagfish heart or even minute fragments taken from either auricle or ventricle fail to contract spontaneously in the absence of external stretch; on the contrary, even the fragments exhibit an amazing degree of rhythmicity, indicating a very diffuse origin of pacemaker activity. This second conclusion pertaining to the pacemaker in these organs is substantiated by applying a tight atrioventricular ligature. This procedure never produced cessation, or even a transient interruption, of the beat in the auricle or the ventricle; the latter chamber merely beats at a slower rate after the ligature is applied (*vide infra*).

As higher filling pressure or increased mechanical tension also will exert a cardioaccelerator effect on the hearts of arthropods, molluscs, amphibians and mammals as well as in the hagfish, it appears that such a mechanism may be quite widespread throughout the animal kingdom. The hagfish heart is, however, aneural indicating that intracardiac nerve influences as mediators in producing this accelerator effect may be considered as not essential in all species.

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If one considers from the standpoint of phylogeny, the extremely primitive biological status of the hagfish in relation to the other chordates, it is interesting to consider the possibility that both myogenic automaticity coupled with a pressure-sensitive accelerator mechanism may represent a very primitive evolutionary development. In this connexion it is noteworthy that the embryonic *Limulus* myocardium exhibits myogenic automaticity, a property which is lost in favor of complete neural control as the cardiac ganglia develop (Carlson & Meek, 1908).

Pharmacologically, the hagfish heart is quite unresponsive to a number of drugs and other compounds, especially acetylcholine and the catecholamines (Fänge & Östlund, 1954; Augustinsson *et al.*, 1956; Jensen, 1957, 1958). However, there is present in the hagfish heart (but not in the liver) a markedly cardioactive material. This substance is not species specific in its action, but also is effective in molluscan (Jensen, 1958) and frog hearts as well as the isolated hypodynamic hagfish heart. In regard to the latter preparation, it may be suggested that the inefficacy of the HHE on freshly isolated hagfish hearts is due to the presence of optimal quantities of the material. During the experiment this is gradually metabolized and/or leached out of the heart, thus rendering it more sensitive to later additions of HHE.

The cardioactive substance derived from hagfish myocardia is not merely cardioaccelerator in the frog but reduces atrioventricular conduction time in that species and will even reverse total atrioventricular block; this point warrants further study.

Regarding the identity of the cardioactive material, little can be stated at present except in general terms. The substance does not appear to be epinephrine. Available data indicate that it is a water-soluble, relatively thermo-stable small molecule. It is apparently not a mineral, protein or lipid. Detailed studies are underway at present toward isolating and characterizing the cardioactive substance.

Two facts are of interest at this point: the liver, the only other large organ in the hagfish body save the gut and muscle mass, possesses no demonstrable

cardioaccelerator activity; second, the heart possesses atypical "secretory" cells. It is at this stage suggestive, if nothing more. It is possible that the hagfish heart may have an endocrine as well as pulsatile function in these primitive animals, and that these atypical cells, located as they are throughout the myocardium, may serve as local "pacemakers" which liberate their secretion upon stretch and concomitant permeability changes.

A brief word regarding the metabolism of the hagfish heart is relevant. Rybak & Boivinet (1959) working on the branchial hearts of the European hagfish or slime eel (*Myxine glutinosa*) found that the auricle : ventricle ratio of $O_2$ consumption in these animals was 1·75 : 1. In the present study, the ratio of heart rate of the entire isolated heart to that of the isolated ventricle alone was 1·63 : 1. (Data from Table 1.) These figures are in surprisingly good agreement, considering the wide differences in technique and even of genera of the fish employed. However, these facts suggest that in the hagfish heart, as in other species, there is a definite metabolic gradient from auricle to ventricle, levels in the former being higher than in the latter, and although this Cyclostome has an extremely high degree of automaticity in all parts of its heart, the graded metabolic rate serves to coordinate its function.

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# STEROL REQUIREMENTS OF A CILIATE *TETRAHYMENA CORLISSI* Th-X

## I. A NUTRITIONAL ANALYSIS OF THE STEROL REQUIREMENTS OF *T. CORLISSI* Th-X*

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## II. METABOLISM OF TRITIATED LOPHENOL IN *T. CORLISSI* Th-X

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**Abstract**—1. *Tetrahymena corlissi* Th-X, a tetrahymenid ciliate, has been cultivated under axenic conditions in a medium of known chemical composition (within limits of contemporary standards of chemical purity) and required a sterol for best growth.

2. It responded to many plant and animal 3β-OH sterols and their esters, with saturated and unsaturated ring systems and variously-substituted side chains.

3. The activity of potential cholesterol precursors was correlated approximately with their presumed proximity to cholesterol; however, tritiated lophenol was not converted to cholesterol or sterols of comparable polarity but was directly incorporated.

4. DL-α-Glycerophosphate, oleic acid and other oleate-containing compounds, and some synthetic phosphatides, spared the sterol requirement and permitted submaximal growth in the absence of sterol.

## I. A NUTRITIONAL ANALYSIS OF THE STEROL REQUIREMENTS OF *T. CORLISSI* Th-X

### INTRODUCTION

THE report that *Paramecium aurelia* required a sterol for axenic growth in a partially-defined medium (Conner & van Wagtendonk, 1955) raised the hope that other ciliates would have similar requirements, since microbes that can be used to assay the biological activity of sterols and sterol precursors are scarce. This expectation has been fulfilled: several species of the genus *Tetrahymena* can be cultured axenically, under conditions where they require a sterol for best growth. We report here on the nature of the requirement of one of them, *T. corlissi* Th-X, a histophagous facultative parasite (Harman & Corliss, 1956; Thompson, 1958) isolated from the circulatory system of a *Pseudotriton* larva (Thompson, 1955).

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## EXPERIMENTAL

Stock cultures of *T. corlissi* Th-X* were grown in a crude medium: Trypticase (Baltimore Biological Lab.) 0·5 g, yeast autolysate (Basamin-Busch; Anheuser Busch Co., Inc.) 0·5 g, dextrin 0·5 g, $K_3PO_4$ 0·1 g, distilled $H_2O$ 100 ml, adjusted to pH 7·0.

To test responses to lipids, the medium in Table 1 was used.

Sterols and terpenes were supplied by the following means: (a) addition of small amounts of concentrated absolute ethanol or propylene glycol solutions to the medium before autoclaving; (b) similar addition of absolute ethanol solutions after autoclaving; (c) addition of water emulsions, formed by injecting concentrated absolute ethanol solutions into warm (65°C) water, before autoclaving; (d) similar addition after autoclaving; (e) addition of concentrated absolute ethanol solutions to sterile culture tubes which were then incubated at 37°C until the alcohol had evaporated, after which autoclaved medium was added; (f) addition of large amounts of dilute propylene glycol solutions to the medium (0·1–1·0 ml per 100 ml) before autoclaving. No striking differences in growth responses were observed when these various methods of sterol presentation were used, except for poorer growth in all cases with method (e). Solvent controls were used. Absolute ethanol alone was slightly stimulatory to growth, propylene glycol was not. Ethanol in excess of 0·2 ml per 100 ml was toxic even when added before autoclaving; see also Vishniac (1955). Propylene glycol inhibited growth at concentrations above 0·5 ml per 100 ml. Water-soluble intermediates were supplied by addition of aliquots of aqueous solutions to the medium before autoclaving. Fatty acids and their esters, and triglycerides, were added as ethyl ether (Spectro grade, Eastman Organic Chemicals) solutions, and spread over the bottoms of the tubes as the ether evaporated, before the medium was added. Phospholipids were supplied from aqueous stock solutions or from aqueous emulsions with the volatile preservative (Table 1).

Sterols were tested alone and in the presence of oleic acid. Other lipids and water-soluble substances were tested alone and in many combinations, in the presence and absence of cholesterol, over wide concentration ranges, from microgram amounts to toxic levels. Serial transfers were used to establish the reproducibility of the effects observed.

For dose-response measurements, stock cultures for inocula were maintained by weekly transfers in the basal medium plus cholesterol 50 $\mu$g per 100 ml. One drop of a 4-day stock culture (optical density 0·35 as measured in the culture tube against a distilled water blank, with a Bausch & Lomb Spectronic 20 spectro-photometer, 530 m$\mu$) was added to each 5 ml of medium. Use of ciliates without depletion or washing did not result in high blanks.

For experiments on the metabolism of labeled 4$\alpha$-methyl-$\Delta^7$-cholestenol (described in Part II), *T. corlissi* Th-X was grown in the medium in Table 1, plus tritiated 4-$\alpha$-methyl-$\Delta^7$-cholestenol (100 $\mu$g per 100 ml) and unlabeled

* Obtained from Dr. Jesse C. Thompson Jr., Hollins College, Virginia.

L-α-(dioleoyl)-phosphoryl ethanolamine (500 μg per 100 ml), in 2500 ml low form culture flasks (Corning 4422) containing 500 ml of medium and covered

Table 1—Basal medium for the cultivation of *T. corlissi* Th-X with lipids

| Component | Final concentrations; Wt. per 100 ml of medium | Component | Final concentrations; Wt. per 100 ml of medium |
|---|---|---|---|
| | (mg) | | (μg) |
| DL-Alanine | 30 | *d*-Panthothenate, Ca | 75 |
| L-Arginine·HCl | 30 | Thiamine·HCl | 50 |
| L-Asparagine·$H_2O$ | 20 | Riboflavin-5′-phosphate, Na | 45 |
| L-Glutamic acid | 40 | Pyridoxamine·2HCl | 5 |
| L-Glutamine | 10 | Pyridoxal·HCl | 5 |
| Glycine | 40 | Biotin | 0·1 |
| L-Histidine·HCl·$H_2O$ | 20 | DL-6-Thioctic acid | 1 |
| L-Isoleucine | 20 | Folic acid | 1 |
| L-Leucine | 20 | | (mg) |
| L-Lysine·HCl | 20 | $K_3PO_4$ | 50 |
| DL-Methionine | 30 | $MgSO_4·7H_2O$ | 50 |
| DL-Phenylalanine | 30 | $CaCO_3$ | 7·5 |
| L-Proline | 20 | Citric acid·$H_2O$ | 60 |
| DL-Serine | 30 | | (μg) |
| DL-Threonine | 40 | Fe | 270 |
| L-Tryptophan | 15 | Zn | 100 |
| DL-Valine | 20 | Mn | 40 |
| $Na_3$guanylate·$4H_2O$ | | Cu | 8 |
| (2′ and 3′) | 10 | Co | 10 |
| Uracil | 4 | Mo | 1·2 |
| | (μg) | | (g) |
| Nicotinic acid | 90 | Glucose | 0·5 |

The amino acids and guanylate were dissolved in hot distilled $H_2O$ (Sol. No. 1), and uracil in a few millilitres of conc. KOH (Sol. No. 2). Vitamins were pooled from conc. stock solutions (Sol. No. 3), Citric acid was dissolved in distilled $H_2O$ and the $CaCO_3$ added (Sol. No. 4). Solutions Nos. 1–4 were mixed and distilled $H_2O$ added to bring the volume to slightly less than half the final volume. Trace metals were added to this solution from a stock solution (0·5 ml per 100 ml medium) of the following composition:

| | |
|---|---|
| $Fe(NH_4)_2(SO_4)_2·6H_2O$, 284 mg; | $CuSO_4·5H_2O$, 6 mg; |
| $ZnSO_4·7H_2O$, 90 mg; | $Co(NO_3)_2·6H_2O$, 10 mg; |
| $MnSO_4·4H_2O$, 32 mg; | $(NH_4)_6Mo_7O_{24}·4H_2O$, 2 mg; |

dist. $H_2$0,100 ml (solution adjusted to pH 2·0 with concentrated HCl). $MgSO_4·7H_2O$ and $K_3PO_4$ were added from stock solutions or as salts. The pH was adjusted to 6·8 with KOH pellets. Distilled $H_2O$ was added to bring the volume to exactly half the final volume. This double-strength (2×) medium, and stock solutions, were stored at 5°C for weeks with a volatile preservative (chlorobenzene, 1,2-dichloroethane, 1-chlorobutane, 1 : 1 : 2, v/v, modified from Hutner & Bkjernes (1948)) added until immiscible globules remained at the bottom of the container. When variables were to be tested in an experiment, 2× medium was dispensed to tubes (2·5 ml per tube), variables were added, and the volume made up to 5 ml with $H_2O$. The medium was autoclaved at 15 lb (121°C) for 30 min. Glucose was added aseptically, with shaking, to the autoclaved medium (~1 drop per 5 ml) from a 40 per cent solution, which had been acidified and autoclaved separately. Cultures were grown at 23–25°C in 20×150 mm tubes covered with aluminium caps and slanted in racks with dividers (Baltimore Biological Laboratories).

TABLE 2—GROWTH OF *T. corlissi* Th-X WITH POTENTIAL STEROL PRECURSORS, STEROLS AND STEROID HORMONES

| Compound | Concentration per 100 ml | Growth*; 7 days |
|---|---|---|
| | (g) | |
| No add | — | 0 |
| Ethyl alcohol | 0·2 | 1 |
| Propylene glycol | 0·2 | 0 |
| | (mg) | |
| Na acetate·$3H_2O$ | 20 | 0 |
| $\beta$-Hydroxy-$\beta$-methyl glutaric acid | 5 | 0 |
| Mevaldic acid | 1 | 0 |
| Mevalonic acid | 1 | 0 |
| Geraniol | 2 | 1 |
| Linalool | 2 | 1 |
| Farnesol | 2 | 1 |
| Squalene | 2 | 1 |
| | ($\mu$g) | |
| Lanosterol | 100 | 1 |
| 24,25-Dihydrolanosterol | 100 | 1 |
| $\Delta^7$-4,4-Dimethylcholestenol | 100 | 1 |
| $\Delta^8$-4,4-Dimethylcholestenol | 100 | 1 |
| $\Delta^{8(14)}$,4-Dimethylcholestenol | 100 | 1 |
| Citrostadienol | 100 | 1 |
| 4$\alpha$-Methyl-$\Delta^7$-cholestenol | 100 | 3 |
| 4$\alpha$-Methyl-$\Delta^8$-cholestenol | 100 | 1 |
| Zymosterol | 100 | 2 |
| Desmosterol | 100 | 4 |
| Desmosteryl benzoate | 100 | 3 |
| $\Delta^7$-Cholestenol | 100 | 3 |
| $\Delta^7$-Cholestenyl acetate | 100 | 4 |
| 7-Dehydrocholesterol | 100 | 4 |
| Cholesterol | 100 | 4 |
| Cholesteryl acetate | 100 | 4 |
| Cholesteryl oleate | 100 | 3 |
| Cholesteryl palmitate | 100 | 3 |
| Cholestanol | 100 | 4 |
| 24-Methylene cholesterol | 100 | 4 |
| Ergosterol | 100 | 4 |
| Stigmasterol | 100 | 4 |
| $\beta$-Sitosterol | 100 | 4 |
| Sitosteryl acetate | 100 | 4 |
| Brassicasterol | 100 | 4 |
| Brassicasteryl acetate | 100 | 4 |
| Fucosterol | 100 | 4 |
| Clionasterol | 100 | 4 |
| Neospongesterol | 100 | 4 |
| Poriferasterol | 100 | 4 |
| Campesterol | 100 | 4 |
| Campesteryl acetate | 100 | 4 |
| Progesterone | 100 | 0 |
| Estradiol | 100 | 0 |
| Testosterone | 100 | 0 |
| Cortisone alcohol | 100 | 0 |

* Numbers indicate optical density ranges obtained in a number of experiments: 0 = 0–0·10, 1 = 0·10–0·20, 2 = 0·20–0·35, 3 = 0·35–0·50, 4 = 0·50–0·75, 5 = 0·75–0·90, 6 = 0·90–1·0, 7 = 1·0–1·5 (Bausch & Lomb Spectronic 20 spectro-photometer, 530 m$\mu$, distilled $H_2O$ blank, tube diameter 20 mm).

with stainless steel closures. The sterol was added to the medium in propylene glycol (final concentration of propylene glycol in the medium 0·2 g per 100 ml). The phosphatide was added as an emulsion in water and the volatile preservative. Flasks were inoculated with 10 ml each of a 7-day culture on the same medium (unlabeled sterol used). Incubation was at 24°C for 7 days. Ciliates were harvested and washed (distilled water) by centrifugation and immediately frozen with dry ice.

## RESULTS

Responses to sterols and potential sterol precursors are summarized in Table 2. Water-soluble substances known or suspected to be intermediates in squalene

Table 3—Growth of *T. corlissi* Th-X with various lipids and lipid components, with and without cholesterol

| Compound | Concentration per 100 ml | Growth*; 7 days | |
|---|---|---|---|
| | | No add | Cholesterol 50 μg per 100 ml |
| | (mg) | | |
| No add | — | 0 | 4 |
| Oleic acid | 1–10 | 1 | 5 |
| Triolein | 1–10 | 1 | 5 |
| Methyl oleate | 1–10 | 0 | 4 |
| Linoleic acid | 1–10 | 0 | 0 |
| Linolenic acid | 1–10 | 0 | 0 |
| Palmitic acid | 1–10 | 0 | 4 |
| Tripalmitin | 1–10 | 0 | 4 |
| Methyl palmitate | 1–10 | 0 | 4 |
| L-α-(Dioleoyl)-phosphoryl ethanolamine | 5–10 | 3 | 5 |
| L-α-(Dipalmitoyl)-lecithin | 5–10 | 2 | 4 |
| L-α-(Dipalmitoyl)-phosphoryl ethanolamine | 5–10 | 1 | 4 |
| L-α-(Dimyristoyl)-lecithin | 5–10 | 1 | 4 |
| L-α-(Dimyristoyl)-phosphoryl ethanolamine | 5–10 | 1 | 4 |
| Tween 20 | 50–200 | 0 | 1 |
| Tween 40 | 50–200 | 1 | 2 |
| Tween 60 | 50–200 | 2 | 5 |
| Tween 80 | 50–200 | 4 | 6 |
| Tween 85 | 50–200 | 3 | 6 |
| | (μg) | | |
| Choline·Cl | 20–100 | 0 | 4 |
| 2-Amino ethanol | 20–100 | 0 | 4 |
| Inositol | 20–100 | 0 | 4 |
| Phosphocholine·Cl (Ca) | 1 μg–1 mg | 0 | 4 |
| | (mg) | | |
| Glycerol | 10–100 | 0 | 4 |
| DL-α-Glycerophosphate (Na) | 50–200 | 4 | 6 |

* See footnote to Table 2.

synthesis were inactive. Terpenes, lanosterol, 24,25-dihydrolanosterol (Kandutsch & Russell, 1959), and three dimethyl sterols (Gautschi & Bloch, 1958) were slightly active. 4α-methyl-$\Delta^7$-cholestenol [lophenol (Djerassi *et al.*, 1958), methostenol (Wells & Lorah, 1960)] showed moderately high nutritional activity while other monomethyl sterols [4α-methyl-$\Delta^8$-cholestenol (Kandutsch & Russell, 1960a, b), citrostadienol (Mazur *et al.*, 1958)] were relatively inactive. A wide variety of 3 β-OH sterols and their esters, from plants and animals, were moderately to highly active. Saturation of the ring system did not cause inactivation and substitutions in the side chain had little effect, but side chain removal, as in the steroid hormones, destroyed activity. Oleic acid increased growth in the presence of all the active sterols.



TABLE 4—GROWTH OF *T. corlissi* Th-X WITH VARIOUS COMBINATIONS OF LIPIDS

| Compound | Concentration per 100 ml | Growth*; 7 days | |
|---|---|---|---|
| | | No add | Cholesterol 50–100 μg per 100 ml |
| Cholesterol | (μg) 50–100 | 4 | — |
| Oleic acid (OLA) | (mg) 1–5 | 1 | 5 |
| DL-α-Glycerophosphate (GP) | 50–200 | 4 | 6 |
| L-α-(Dipalmitoyl)-lecithin (DPL) | 6–10 | 2 | 4 |
| L-α-(Dioleoyl)-phosphoryl ethanolamine (DOC) | 0·5–5 | 3 | 5 |
| OLA + DPL | — | 3 | 6 |
| OLA + GP | — | 4 | 6 |
| OLA + DPL + GP | — | 6 | 7 |
| DOC + DPL | — | 4 | 5 |
| DOC + GP | — | 5 | 6 |
| DOC + DPL + GP | — | 6 | 7 |
| DPL + GP | — | 4 | 5 |

*See footnote to Table 2.

Of the other lipids and lipid components tested (Table 3), DL-α-glycerophosphate, oleic acid and oleate-containing compounds (methyl oleate, triolein, L-α-(dioleoyl)-phosphoryl ethanolamine, Tweens 80 and 85) were potent cholesterol-sparers. In the absence of cholesterol, the order of growth-promoting activity was: DL-α-glycerophosphate, L-α-(dioleoyl)-phosphoryl ethanolamine, Tween 80, Tween 85, L-α-(dipalmitoyl)-lecithin, Tween 60,† oleic acids, triolein, the other synthetic phosphatides, and Tween 40.† Other unsaturated fatty acids, palmitic acid and its methyl ester and triglyceride, Tween 20, and other phosphatide

† Contains a small amount of oleic acid.

components were inactive alone and did not spare cholesterol. Cholesterol always stimulated growth in the presence of single lipids and combinations of lipids that permitted growth in the absence of a sterol (Tables 4 and 5).

Table 5—Growth response of *T. corlissi* to cholesterol, with and without oleic acid

| Compound | Concentration ($\mu$g per 100 ml) | Optical density*; 7 days | |
|---|---|---|---|
| | | No add | Oleic acid 5 mg per 100 ml |
| Cholesterol | 200 | 0·50 | 0·74 |
| | 100 | 0·54 | 0·75 |
| | 50 | 0·50 | 0·64 |
| | 40 | 0·41 | 0·58 |
| | 30 | 0·35 | 0·55 |
| | 20 | 0·28 | 0·48 |
| | 10 | 0·16 | 0·35 |
| No add | — | 0·02 | 0·07 |

* Averages of values of triplicate tubes.

Labeled 4$\alpha$-methyl-$\Delta^7$-cholestenol did not appear to undergo metabolic transformation. A complete description of the results of this experiment appears in Part II.

## DISCUSSION

The pattern of response of the ciliate to sterols and potential precursors suggests impairment of the mechanism for sterol synthesis. Unfortunately, we do not know the nature of the sterol(s) of tetrahymenid ciliates. Some species of *Tetrahymena* do not have a nutritional requirement for a sterol. *T. pyriformis* S synthesizes one or more that have been extracted but not identified (Seaman, 1950). A neutral, steroid-like fraction has been isolated from *T. pyriformis* W (McKee *et al.*, 1947), and the same ciliate can supply material which will permit the growth of *Paramecium aurelia* (Conner, 1957); a ciliate reported to have a sterol requirement satisfied by stigmasterol but not by cholesterol (Conner & van Wagtendonk, 1955). It was suggested (Conner, 1957) that the *Tetrahymena* sterol has a molecular configuration like stigmasterol. Inhibition of growth of *T. pyriformis* W by dinitrophenol, colchicine, progesterone, and cortisone derivatives is reported to be reversed by stigmasterol and cholesterol (Conner & Nakatani, 1958; Conner, 1959), and acetate and $\beta$-sitosterol are active in reversing inhibition caused by eight purine analogs (Dewey *et al.*, 1959), 2,6-diamino-4-isoamyloxypyridine (Markees *et al.*, 1960) and various antimetabolites of acetate (Dewey & Kidder, 1960). Mevalonic acid was ineffective in reversing inhibition by $\alpha$-phenylbutyric, $\gamma$-phenylbutyric and cyclopropane carboxylic acids.

β-Sitosterol and lecithin protected against inhibition by α-biphenylbutyric acid, and β-sitosterol had a slight reversing effect on α-phenylbutyric acid inhibition; cholesterol and squalene were ineffective. Lecithin and an enzymatic digest of vitamin-free casein, with and without acetate, were potent reversing-agents. Fatty acids from saponified lecithin were inactive. It was suggested that acetate antimetabolites prevent formation of a precursor compound having more than one function, and the ineffectiveness of mevalonic acid in reversing inhibition was attributed to inability of the ciliate to activate exogenous mevalonic acid by phosphorylation.

All of the inhibitor actions that are reversed by sterols or sterol-containing crudes (lecithin, casein digest) can be attributed to impairment of sterol synthesis. Dinitrophenol (Loomis & Lipmann, 1948), colchicine (Lettré & Albrecht, 1951) and the steroid hormones (Blecher & White, 1960) probably interfere with the maintenance of an ATP level sufficient to sustain adequate rates of phosphorylations concerned with the metabolism of mevalonic acid. Wright & Loeb (1959, 1960) have shown that in rat liver homogenates, under aerobic conditions, the conversion of mevalonic acid to non-saponifiable materials is a direct function of endogenous ATP concentration, and dinitrophenol and other inhibitors of oxidative phosphorylation block incorporation of mevalonic acid into the non-saponifiable fraction and cholesterol. It is apparent that sterol synthesis by *T. pyriformis* W can be interfered with by a variety of means, some quite indirect. A metabolic basis for this sensitivity is called to mind by the observations that whole yeast cells (Kodiecek, 1959) and a mold, *Carpenteles brefeldianum* (Glover & Leat, 1957), can incorporate acetate into sterols preferentially.

We consider it likely that cholesterol is the principal sterol of *T. corlissi* Th-X, or that it is a key intermediate in reactions leading to the formation of ciliate sterol. Vishniac (1947) has found it in a *Labyrinthula* species, and it composes 30 per cent of the non-saponifiable fraction of *Trypanosoma cruzi* (von Brand *et al.*, 1959). Since ciliates are only distantly related to these protists (though all are classified as Protozoa by protozoologists), this comparison may be meaningless. The nutritional activity of the sterols that have been suggested as precursors of cholesterol [4α-methyl-$\Delta^7$-cholestenol (Wells & Lorah, 1960), zymosterol (Johnston & Bloch, 1957), desmosterol (Stokes & Fish, 1960) and $\Delta^7$-cholestenol (Frantz *et al.*, 1959)] may indicate that they can function as intermediates in the synthesis of ciliate sterol, and/or can be incorporated directly in place of the normal sterol. Molecular configuration would be critical in determining ability to substitute by direct incorporation. The other active sterols that are probably not intermediates in cholesterol synthesis may be converted to ciliate sterol and/or incorporated directly.

The role(s) of sterols in ciliates and other protists are largely unknown. Precedent from work on metazoan cells suggests that there are multiple roles; structural and metabolic. There is little doubt that sterols are important components of all membranous interfaces in protists, except bacteria. Metabolic functions like those described for cholesterol in metazoans (precursors of steroid hormones, bile

salts and vitamin D) are not known, but a sterol from a slime mold has been reported to have acrasin activity (Heftman *et al.*, 1959), and the presence of cholesterol in purified cytochrome oxidase (Marinetti *et al.*, 1957) shows the intimate nature of the involvement of this sterol in the activity of a biological catalyst widespread among protists.

The nutritionally-active sterols for the ciliate may be acting in dual capacities, serving structural and metabolic needs. Clark & Bloch (1959a,b) have proposed such a two-fold function for sterols fed to insects. They found that cholesterol could meet both demands in *Dermestes vulpinus*. A small amount (3 per cent of the total) was absolutely required for growth (metabolic requirement), other sterols satisfied the remaining large fraction of the demand (structural requirement). Cholestanol, $\Delta^7$-cholestenol, 22-dehydrocholesterol, 22-dihydroergosterol, $\Delta^7$-ergosterol, β-sitosterol, stigmasterol, 22-dihydroergosterol and $\Delta^{7,22}$-ergostadienol spared cholesterol but failed to replace it. Ergosterol, lanosterol, $\Delta^8$-4,4-dimethylcholestenol, squalene and mevalonic acid were inactive. The bulk of the cholestanol fed as a sparing sterol was recovered unchanged. Desmosterol and 7-dehydrocholesterol replaced cholesterol completely, indicating conversion by terminal hydrogenases. Clark & Bloch's hypothesis cannot be applied in all details to the ciliate situation. *T. corlissi* Th-X does not have an absolute micro-requirement for cholesterol. One must postulate a small conversion of all nutritionally-active sterols to cholesterol, or a limited total synthesis of cholesterol. As is discussed below, either postulate is consistent with the facts; the second is the more attractive.

The molecular configuration requisite to direct incorporation of a sterol is not obvious. Very small structural differences sharply affect nutritional activity. Of the monomethyl sterols tested, only 4α-methyl-$\Delta^7$-cholestenol was active. 4α-methyl-$\Delta^8$-cholestenol, which differs only in the position of the double bond in the ring system (like the utilizable zymosterol), and citrostadioenol (4α-methyl-$\Delta^7$, 24(28)-stigmastadienol), which differs only in the possession of a 24-ethylene group (like the utilizable fucosterol), were inactive. A wide variety of other sterols with different side chain structures and degrees of saturation of ring A were active. Some of these [7-dehydrocholesterol (Fraenkel *et al.*, 1941; Beck & Kapadia, 1957), β-sitosterol (Bergmann & Levinson, 1958), ergosterol (Clark & Bloch, 1959c)] have been shown to be convertible to other sterols in insects. In support of direct incorporation are the observations of : (a) the utilization of cholestanol, a sterol which is not likely to be converted to ciliate sterol, and (b) the failure to convert tritiated 4α-methyl-$\Delta^7$-cholestenol to detectable amounts of more polar sterols (see Part II). Since 4α-methyl-$\Delta^7$-cholestenol met the sterol requirement alone, some slight (and undetected) conversion of it to cholesterol may have occurred, if performance of the metabolic role requires a higher degree of structural specificity than performance of the structural role. Alternatively, a small amount of cholesterol may have been synthesized.

The lack of activity of squalene, mevalonic acid and β-hydroxy-β-methyl glutaric acid, compounds whose status as intermediates in cholesterol synthesis is

unquestioned, indicates that the locus of impairment of synthesis is between lanosterol and ciliate sterol. Lanosterol would be nutritionally-active, even if it could not be converted to ciliate sterol, if it could be incorporated and perform the structural function. Apparently its shape precludes this.

The inactivity of compounds without the side chain (sex hormones, cortisone), and the activity of sterol esters, have been reported for other protists (Smith, 1959) and insects (Levinson & Bergman, 1957). Non-specific esterase activity has been observed during digestion in *T. corlissi* strain W (Müller *et al.*, 1960).

Since it responds to cholesterol, *T. corlissi* Th-X differs from *Paramecium*, the other sterol-requiring holotrichous ciliate that has been studied (Conner & van Wagtendonk, 1955). In this respect it resembles various *Trichomonas* species (Shorb & Lund, 1959; Cailleau, 1936, 1938; Sanders, 1957), *Labyrinthula vitellina* var. *pacifica* (Vishniac, 1955), *Peranema trichophorum* (Storm & Hutner, 1953) and pleuropneumonialike organisms (Edward & Fitzgerald, 1951; Rodwell, 1956; Butler & Knight, 1960; Smith, 1960). It is possible that this characteristic is related to its phagotrophic and facultative parasitic habits.

Growth of the ciliate in the absence of sterol, with DL-α-glycerophosphate and sources of oleic acid, depends upon the effectiveness of these compounds in supporting sterol synthesis; directly as precursors, or indirectly as participants in some other way. The synthesis of sterol for structural purposes could not be spared by compounds simpler than those which can satisfy the demands of the architecture of the membrane structure. However, glycerophosphate and oleic acid may spare the need for sterol for metabolic purposes if they are products of sterol-dependent reactions. L-α-Glycerophosphate is a key intermediate in the synthesis of phosphatidic acid, glycerophosphatides, and di- and tri-glycerides (Rossiter & Strickland, 1959; Weiss *et al.*, 1960). It may be formed by the action of glycerophosphate dehydrogenase on dihydroxyacetone phosphate from glycolysis and/or by acylation of glycerol. There is no precedent for its direct participation as a substrate in sterol synthesis, however. Labeled non-saponifiables were not reported as products of enzymatic conversions of $C^{14}$-labeled L-α-glycerophosphate (Stein & Shapiro, 1957; Rossiter & Strickland, 1959). The inactivity of glycerol for *T. corlissi* Th-X implies an inability to carry out the glycerol kinase reaction. The responsible enzyme has been reported to be absent from the intestinal mucosa of the pig (Buell & Reiser, 1960).

In the case of oleic acid, Kodicek (1959) has reported that $C^{14}$-fatty acids from yeast were incorporated by intact yeast cells, under anaerobic conditions, into the carbon of unsaponifiable material to an extent ten times that of $C^{14}$-acetate. He interprets this to mean that some of the fatty acids were incorporated into isoprenoid units without cleavage to 2C fragments. If oleic acid was used in this way by *T. corlissi* Th-X, its sterol-sparing activity would be understandable. To be sterol precursors, L-α-glycerophosphate and oleic acid would have to be incorporated at a level in sterol synthesis beyond mevalonic acid, since mevalonic acid was inactive for the ciliate. The permeation of mevalonic acid into intact cells has been reported to be poor, but incorporation into non-saponifiables is always detected.

Impermeability is probably not the basis for the complete inactivity of mevalonic acid for the ciliate.

It is of interest that sterol requirements in protists are often accompanied by fatty acid requirements or stimulations; anaerobically-grown yeast (Andreason & Stier, 1953), pleuropneumonialike organisms (Smith, 1960), *Paramecium* (Miller & Johnson, 1960), *Trichomonas* (Shorb & Lund, 1959). In some cases, cholesterol appears to act by protecting against the toxicity of the fatty acids. This may be a function of cholesterol when *T. corlissi* Th-X is grown with oleic acid but, since good growth occurs with the sterol alone, it can only be a collateral action. Cholesterol protects against the toxicity of unsaturated fatty acids to Gram positive bacteria, but cholestanol, 7-dehydrocholesterol, ergosterol, zymosterol and sitosterol do not (Kodicek, 1949). These sterols, however, are as active as cholesterol in promoting growth of the ciliate with oleic acid.

Tweens 80 and 85, which contain oleic acid as the predominant fatty acid, were active for the ciliate, but their use in experiments on sterol actions were avoided because they contain traces of unsaponifiables (J. F. Treon, Atlas Powder Co.). Tween 80 has been employed routinely at a concentration of 1 per cent in the cultivation of *T. pyriformis* W; most recently in work on sterol reversal of antimetabolite actions (Conner & Nakatani, 1958; Conner, 1959; Dewey *et al.*, 1959; Markees *et al.*, 1960; Dewey & Kidder, 1960). Though no ambiguities appear to have been introduced by its use in such large amounts, future work of this sort would seem to be done best with Tween-free media.

The nutritional activity of the synthetic phosphatides, in the absence of sterols, may be attributed to their content of glycerophosphate and, except for the dipalmitoyl forms, fatty acids. However, Goldberg & De Meillon (1948) have made the provocative observation that sterol-free egg yolk lecithin replaced cholesterol for larvae of *Aedes aegypti*. Fatty acids and triglycerides were inactive, and a combination of the phospholipid preparation and cholesterol was best. Phosphatides also potentiated the active lipids for *T. corlissi* Th-X (particularly oleic acid), as they do for other protists (Kodicek, 1959; Storm & Hutner, 1953).

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Technological Station, Univ. British Columbia; cholesteryl oleate, Dr. S. H. Hutner, Haskins Lab.; brassicasterol, clionasterol, neospongesterol, poriferasterol, campesteryl acetate, Dr. W. Bergmann, Yale University; synthetic phosphatides (except palmitoyl forms) Dr. Erich Baer, University of Toronto; Tweens, J. F. Treon, Atlas Powder Co.; L-α-(dipalmitoyl)-lecithin and phosphoryl ethanolamine, β-hydroxy-β-methyl glutaric acid, amino alcohols, California Corporation for Biochemical Research; fatty acids, esters, and triglycerides, Hormel Foundation, except oleic acid and methyl oleate, Applied Science Laboratories, Inc.; glycerol, geraniol, linalool, cholesterol, ergosterol, Distillation Products Industries; cholesteryl acetate, sitosteryl acetate, 7-dehydrocholesterol, progesterone, cortisone alcohol, testosterone, estradiol, DL-α-glycerophosphate (Na), Nutritional Biochemicals Corp.; cholestanol, Mann Laboratories; cholesteryl palmitate, Aldrich Chemical Co., farnesol, K and K laboratories.

## II. METABOLISM OF TRITIATED LOPHENOL IN *T. CORLISSI* Th-X

### EXPERIMENTAL

*Preparation of tritiated lophenol*

For tritium labeling, 40 mg of lophenone (4α-methyl-$\Delta^7$-cholesten-3-one, kindly provided by Dr. Carl Djerassi) were dissolved in a mixture of 0·4 ml of ethanol and 0·2 ml of $T_2O$ (20 mc of T) containing 4·3 mg of KOH. The solution was heated under reflux for 1½ hr, cooled and the lophenone allowed to precipitate. After crystallization from absolute ethanol, the tritiated ketone (30 mg) was reduced with 60 mg $LiAlH_4$ in 15 ml of ether. After 2 hr the reaction mixture was worked up in the usual manner and the lophenol purified by chromatography on alumina and recrystallization. The final product had m.p. 146–149°C (reported 149°C) and a specific activity of 7300 c.p.m./μg.

*Isolation of Tetrahymena sterols after feeding of tritiated lophenol*

Approximately 5·5 g (wet wt.) of *Tetrahymena corlissi* Th-X, obtained as described in Part I, were digested by refluxing under nitrogen in 50 ml of methanol–water 1 : 1 containing 10 per cent KOH. The cooled reaction mixture was extracted three times with Skellysolve B, the extracts washed with water and dried over anhydrous sodium sulfate. The residue, 26 mg of a brownish partly crystalline material, had 119 d.p.m./μg or a total of $4{\cdot}95 \times 10^6$ c.p.m. of T. This corresponded to a recovery of 20 per cent. The crude non-saponifiable fraction was chromatographed on a silicic acid–celite (3 : 1) column by gradient elution with Skellysolve B–benzene. A small amount of radioactivity ($0{\cdot}14 \times 10^6$ c.p.m.) was associated with an early, non-polar fraction. Fractions 80–114 (15 ml of eluent solvent per fraction) weighing 8 mg contained $4{\cdot}9 \times 10^6$ c.p.m. (600 d.p.m./μg) or essentially all of the radioactivity on the column. No radioactivity was found in later column fractions, indicating the absence of labeled cholesterol or sterols having similar polarity.

To this material 24 mg of authentic lophenol were added as carrier and the mixture chromatographed on an alumina column (Woelm, activity II, neutral, 0·96 g, 4 × 48 mm). Elution with Skellysolve B–benzene 1 : 1 yielded a main peak (25 mg) having 105 c.p.m./μg. After crystallization the sterol melted at

149–150°C (reported for lophenol 149–151°C) and had 116 c.p.m./$\mu$g. The melting point and the specific activity of the lophenol were not changed by further crystallizations. The identity of this material with lophenol is shown by the data of Fig. 1 in which the weights and the radioactivities of the sterol fraction (containing carrier lophenol) are plotted on the same graph. Correcting for the addition

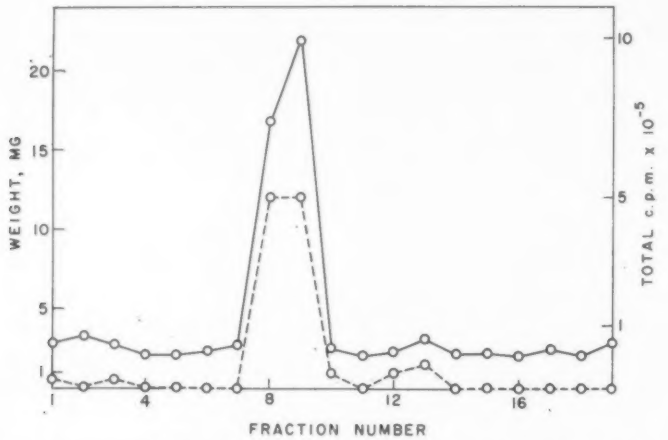


Fig. 1. Chromatography of reisolated radioactive sterol after mixing with authentic lophenol. Solid line, total counts per fraction; dashed line, weights in milligrams per fraction.

of lophenol carrier before alumina chromatography, a specific activity of 150 c.p.m./$\mu$g would have been expected for the purified lophenol. The value found (116 c.p.m.) indicates that the crude sterol fraction (from the first chromatogram) contained at most 23 per cent of radioactive materials other than lophenol. From the above data it can also be calculated that lophenol comprised approximately 20 per cent of the total non-saponifiable fraction from this experiment. The remainder has not been characterized.

## DISCUSSION

*Tetrahymena corlissi* Th-X raised on T-labeled lophenol incorporated a significant amount of the sterol from the medium. Analysis of the non-saponifiable fraction isolated from the cells revealed that essentially all of the incorporated radioactivity was present in the form of lophenol itself. Metabolic products of lophenol such as cholesterol or sterols of comparable polarity could not be detected. It is, however, not possible to exclude formation of such products entirely because lophenol was labeled with tritium in potentially unstable positions. For example, on dehydrogenation of lophenol to lophenone, the tritium at the carbon atoms adjacent to the carbonyl group (C–2 and C–4) might become susceptible to exchange by enolization. However, at physiological pH and temperature, exchange

reactions of this type are unlikely to be extensive. Moreover, we have previously employed the same labeling technique for introducing tritium into sterol intermediates and have shown, for example, that in the biological demethylation of lanosterol metabolites hydrogen attached to C–2 is largely retained (F. Gautschi & K. Bloch, unpublished experiments). On the basis of this experience it seems reasonably certain that the absence of labeled cholesterol or other demethylated products in the present experiments is due to the metabolic inactivity of lophenol and is not caused by the loss of tritium from intermediary products. It thus appears that the growth-promoting activity of lophenol for the ciliate is not associated with any metabolic transformations. Lophenol appears to satisfy the sterol requirement of this organism by entering the cell structure unchanged, in a manner similar to the cholesterol-sparing action exhibited by cholestanol and β-sitosterol in the nutrition of sterol-requiring insects.



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**SHORT COMMUNICATION**

# THE OXIDATION OF GLUCOSE BY CRUSTACEANS*

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**Abstract**—Crabs (*Hemigrapsus nudus*) utilize glucose as an oxidative substrate in all stages of the intermoult cycle, as shown by isotopic labelling experiments. Simultaneous administration of unlabelled glucose, with the labelled sugar, greatly decreases the proportion of $^{14}C$ in the respiratory $CO_2$.

SOME years ago, one of us suggested that glucose is not used as a substrate for oxidative metabolism in crustaceans (Scheer & Scheer, 1951). This suggestion was based on the observations that glucose does not stimulate oxygen consumption of surviving tissue fragments, and that no radioactivity could be detected in the respiratory $CO_2$ of lobsters after injection of $^{14}C$ glucose. Subsequently, Hu (1958), in this laboratory, was able to demonstrate oxidation of glucose by crabs with a more refined isotopic technique. A. W. Pritchard (personal communication) has made similar observations with crayfish. It has been suggested by Vonk (1960) that our earlier results may be accounted for by a high level of oxidation of endogenous substrates. There was also the possibility that utilization of glucose as a substrate for oxidative metabolism varies during the stages of the intermoult cycle. This study explores both of these possibilities.

We have utilized the shore crab *Hemigrapsus nudus*, collected near Charleston, Oregon, and maintained in aquaria at 10–15°C in shallow sea water. Stage in the intermoult cycle was determined by the method of Kincaid & Scheer (1952). Glucose uniformly labelled with $^{14}C$ was injected into the ventral sinus through a leg joint. During an experiment, the animals were maintained in small containers of artificial $CO_2$-free sea water through which a stream of $CO_2$-free air was aspirated. The $CO_2$ was collected in $CO_2$-free NaOH during the experiment. After each collection period, the animal was removed, HCl was added, and the dissolved $CO_2$ aspirated into the NaOH. The combined $CO_2$ was precipitated as $BaCO_3$ and plated and counted by the method used by Hu (1958), with correction for self-absorption.

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For studies of the relation of glucose oxidation to stage in the intermoult cycle, crabs were injected with 0·2 $\mu$c of $^{14}C$ glucose, with 0·5 mg of carrier glucose. The $CO_2$ was then collected for periods of 12–36 hr. The results are given in Table 1. It is clear that glucose is oxidized in all stages of the cycle, including the postmoult period (B) when chitin synthesis is occurring (Meenakshi & Scheer, 1961) and the

TABLE 1—OXIDATION OF GLUCOSE IN RELATION TO STAGE IN THE INTERMOULT CYCLE IN *Hemigrapsus nudus*

| Sex | Stage | Wt. (g) | Duration of collection (hr) | Radioactivity in $CO_2$ (c.p.m.) | Injected $^{14}C$ recovered in $CO_2$ (%) |
|---|---|---|---|---|---|
| F | $B_1$ | 12·8 | 24 | 5844 | 15·3 |
| M | $B_2$ | 18·6 | 18 | 4099 | 10·7 |
| F | $C_1$ | 17·6 | 12 | 1655 | 4·3 |
| M | $C_1$ | 26·4 | 18 | 5674 | 14·8 |
| M | $C_1$ | 14·5 | 18 | 2736 | 7·2 |
| F | $C_3$ | 14·9 | 18 | 5924 | 15·4 |
| M | $C_4$ | 16·5 | 12 | 597 | 1·6 |
| M | $C_4$ | 19·8 | 24 | 7358 | 19·2 |
| M | $C_4$ | 14·8 | 24 | 3623 | 9·5 |
| M | $C_4$ | 12·5 | 36 | 6147 | 16·7 |
| M | $D_1$ | 15·0 | 24 | 1292 | 3·3 |
| M | $D_2$ | 18·7 | 24 | 2859 | 7·8 |

TABLE 2—EFFECT OF GLUCOSE ADMINISTRATION ON RECOVERY OF LABELLED GLUCOSE FROM RESPIRATORY $CO_2$ OF *Hemigrapsus nudus*

Values are counts per minute in respiratory $CO_2$

| Treatment | Collection period (hr) | | | | | |
|---|---|---|---|---|---|---|
| | 1–12 | 13–24 | 25–36 | 37–48 | 49–60 | 61–72 |
| Starved | 1658 | 2004 | 418 | — | — | — |
| 10 mg glucose 3 hr before injection | 1763 | 783 | 761 | 590 | 289 | 416 |
| 10 mg glucose at time of injection | 322 | 263 | 608 | 686 | 687 | 725 |
| 10 mg glucose at time of injection and every 12 hr | 382 | 289 | Trace | 0 | 0 | 0 |

late intermoult and early premoult ($C_4$–$D_1$) when glycogen synthesis is vigorous (Renaud, 1949; Scheer, 1959). It is also clear that there is an individual variation in the intensity of this oxidation not related to sex or moult cycle.

One of the factors involved in this variation is the simultaneous oxidation of other C sources, as shown by the experimental results in Table 2. Administration

of 10 mg of glucose with the labelled glucose greatly decreased the proportion of $^{14}C$ in the respiratory $CO_2$, as compared with the proportion in $CO_2$ from an animal given no carrier glucose, or one given glucose several hours before the labelled sugar. Administration of unlabelled glucose at regular intervals with and after the labelled sugar results in almost complete "sparing" of the labelled glucose.

We therefore conclude that crabs use glucose as a substrate for oxidative metabolism throughout the intermoult cycle. Previous failure to observe this utilization in spiny lobsters was probably due to the techniques used, and to the nutritional state of the animals used.

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*Comp. Biochem. Physiol.*, 1961, *Vol.* 2, *pp.* 221 *to* 229. *Pergamon Press Ltd., London. Printed in Great Britain*

# A RUSSIAN VIEW ON REGENERATION IN ANIMALS

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A review of *Asexual Propagation and Regeneration* by M. A. Vorontsova and L. D. Liosner, Pergamon Press, London, 1960. pp. xxiv + 489, 70s.

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THIS translation of the authors' *Bespoloe Razmnojenie i Regeneratsiya* will be welcomed by all those English-speaking students of the subject who do not have access to the original, Russian edition or who have difficulty with the language. It contains a wealth of information, much of which comes from Russian, and other, original papers not easily available here, and not widely known. It illustrates the type of concentrated information which offers best returns for translation-labour.

We are indebted to the Pergamon Press for their enterprise in this work and particularly for their public-spirited policy of paying royalties to the Russian authors. They hope that this may encourage the Soviet Authorities to accord reciprocal recognition to western authors interested in the brisk translation service in the opposite direction. In view of this, public-spirited biologists, and certainly those who have experienced the generosity of Russian authors, will not consider the price of this book excessive.

The English translation is a photographic reproduction of typescript and suffers from the practical and aesthetic drawbacks of such a format, but this is a minor matter and the publishers give reasons why this was justified in the interests of time and expense. The original edition appeared in 1957 and it was reasonable to avoid undue delay in translation. The original date should be borne in mind, so as to avoid blaming the authors for omitting more recent results. At the same time the advertisement on the jacket is scarcely justified in claiming that the book includes the latest facts on the subject, which has expanded considerably in the last 3 years.

Some of the illustrations are better than in the original because of the better quality paper but some have lost as much as they have gained, or more, through a further reduction to 75 per cent of the linear measurements. This is a pity because some were already very small.

The book is primarily a study of reparative regeneration, and the subjects asexual reproduction and physiological regeneration are considered only for their resemblances to regeneration proper. It is therefore debatable if they should have been dealt with before regeneration. For another reason also asexual reproduction is not ideal for the first section: the account is largely of well-established descriptive knowledge, in contrast to the many new experimental results in the field of

regeneration. The study of asexual reproduction at present adds little to the analysis of the mechanisms of regeneration and, significantly, it plays little part in the philosophical discussions of the last chapter until specifically broached in the last section. The study of physiological regeneration also adds little because it is related only logically to reparative regeneration, i.e. because it makes good certain losses, and not in its actual procedure.

Within the section on regeneration proper there is much more information on the Amphibia than on any other group; this is a reflection of present knowledge and is only partly due to the authors' own work being mainly on the Amphibia. The focus of interest in the book is on the results from this group and from here it attenuates progressively through regeneration in other groups and on to the related processes. Partly because of the relatively small amount of information from other groups it might have been preferable simply to add this to the results from Amphibia. Its corroborative or contrasting significances in fact would show to best advantage thus.

In any event it is questionable if a taxonomically-arranged presentation is the best for this kind of subject matter. In most subjects except comparative anatomy, facilities for rapid comparisons of particular processes across the whole taxonomic field are necessary and the logical arrangement is by processes and topics, i.e. by subject matter, rather than by taxonomic groups. This of course is not to deny that in special cases, such as the incidence and distribution of regenerative powers among animals, taxonomic position is the centre of interest. Since in works of this kind space does not permit even an outline of the comparative anatomy and systematics of the groups there is no real reason for tying the material to a taxonomic framework. Any such compulsion inevitably leads to overlap and repetition of subject matter and ideas, particularly evident in the section on asexual reproduction, while on the other hand valuable comparisons and generalizations may be overlooked. Under these circumstances an extensive system of page cross-references would have been helpful. For instance, a reference to the same phenomenon in *Hydra* (p. 173) would have strengthened the evidence that in Amphibia non-irradiated grafted tissues are capable of restoring regenerative power to irradiated indigenous tissue. Another example is the influence of the nerve supply on regeneration, in various groups.

The treatment, both in the Amphibia and in other groups, is largely restricted to the morphogenetic aspect, in which the authors themselves have been most interested, and there is little indication of recent work on biochemical and physiological changes, locally or systematically, during regeneration. No comprehensive review in English of the results of Russian workers in this field has appeared since the section in Joseph Needham's book, *Biochemistry and Morphogenesis*, and a recent survey would have been very welcome. To judge by the early promise, we should anticipate advances during the interim as great as those in other countries.

In the morphogenetic field the outstanding recent achievements of the authors and other Russian workers is the analysis of the role of specific tissues in the

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regeneration of the external appendages of Amphibia. A clear picture emerges of the inductive and organizing capacity of each of the main tissue-systems of the stump, skin, muscle and skeleton. Muscle has the greatest power, but under experimental conditions all can produce far more than their prospective fates in a normal regenerate, whether by the proliferation of the cells they donate (Umanski) or by their inducing action on the host tissues (Vorontzova & Liosner). The relative importance of the two is still uncertain. The work of Trampusch in Holland might have been added to this section. Longitudinal halves of a limb stump similarly regenerate distally more than their prospective achievement in an undivided stump. Fractions of embryonic organisers show similar expansive potencies.

As indicated above, the work on tissue-grafts has also shown that normal tissues can restore regenerative ability to irradiated tissues and this may necessitate some re-appraisal of earlier results using irradiated tissues. It still seems probable that such tissues do not recover the power without outside help and it is possible that in some animals migrant "neoblasts" may be adequate for this. It has usually been supposed that the latter provided only their own activity.

The authors object to the classical terms "induction", "organization" and "determination" (pp. 127, 178, 203, 356), and indeed they are confirmed rebels at almost every turn. In this case their justification is questionable: if it becomes necessary to use the alternatives that one tissue is "implicated" by another (p. 127) or has its "differentiation deflected" by this (p. 356), or "adapts itself" to this (p. 355) then there has been a loss rather than a gain in clarity and specificity, and new terms have been added which in turn may never have more than a hallowed aura of real meaning. In fact at present the classical terms probably define the phenomena we envisage as precisely as they can be defined, at least for the latin-based languages.

The objection to "blastema" (p. 311) is shared by some American workers, and has some justification on their showing. However, again it is necessary to use a cumbersome and less specific alternative "regeneration-bud". It would be simpler to retain and redefine "blastema". Certainly there is regenerative activity in the stump also, but this does not reduce the reality of the blastema.

The authors believe (p. 333) that the results of tissue grafting and other work discredit Weiss and Guyenot's classical idea of regeneration-"fields", within each of which regenerates are always completely typical. In fact it has long been clear that the regenerate can be *quantitatively* abnormal, e.g. under experimental conditions, while on the other hand the present results continue to confirm the *qualitative* specificity of each region. In view of Kiortsis' further demonstration of "neutral" regions between active fields and of other recent work, there seems no good reason to abandon the conception of fields because (like embryonic fields) they eventually develop regional autonomy. Sometimes two fields adjoin and a hybrid regenerate may be obtained at the boundary. As the authors point out (p. 266) the serial heteromorphs of Crustacea are often those of a distant segment, so that the overlap of two fields will not explain such cases.

Some new evidence is provided on another leading problem, the stage at which a blastema becomes determined for self differentiation. The authors decide that it is probably determined from the outset but their evidence that grafts of an isolated tissue can change the fate of some of the host tissues strongly implies that all the tissues of the latter, together and in their normal positions, are likely to determine fully, or even to redetermine, a small grafted blastema. Inducing activity is largely proportional to the relative masses of inducing and induced tissues.

Other new contributions described include an extension of work on the regeneration of structures defective as a result of operations in the embryonic stage. These often continue to regenerate with precisely the same defect, though in some cases there is a progressive improvement in successive acts of regeneration. Recent work on the subject by Newth was not then available. Another significant conclusion (p. 300) is that there is no absolutely "dead" period between the time when embryos restore their form by regulation and the time when larvae begin to do so by true epimorphic regeneration. The authors appear to support Svetlov's idea that there is merely a period when regenerative power is rather low because of competition from the rapid normal growth of the late embryo. Nevertheless the whole mode of replacement changes and therefore there is a functional discontinuity at least.

Recent Russian work has further established the importance for the initiation of regeneration in external organs of *both* trauma and a subsequent cover of migrated epidermis. However, trauma alone, without an open wound *can* initiate, just as it must in internal organs. Work on regeneration in experimentally inverted limbs is described. Work on this by E. G. Butler could have been included with advantage. Much of the recent work on dedifferentiation by Butler's group and also by Thornton also was omitted and it seems that the authors did not have access to much of the recent work from other laboratories, including those of Schotté, Singer, Lehmann, Wolff and Guyenot.

These were not the only new contributions on Amphibian regeneration. Some aspects however were not up to date. For instance, the authors were unnecessarily pessimistic, even then, about available knowledge on mitosis rates and times. Since then further information has become available from Chalkley, Green *et al.*, and others. The authors' interest in their own field perhaps makes it particularly difficult to do full justice to all stages and aspects of the process of regeneration.

As the advertisement claims, a considerable section is devoted to regeneration in the mammals. It adds considerably to general knowledge of the powers of regeneration in the various organs, and emphasizes the great ability of many internal organs. The authors therefore contend that regenerative power does not decline with increasing differentiation of tissues and even that the power in internal organs may actually have increased during evolution (p. 410). However, it should be emphasized that the power to regenerate limbs, nerve centres and other organs manifestly does show a general decline in the higher animals. Moreover, the lower Metazoa have also the greater ability to regenerate internal organs, as witness the

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gut of holothurians, and of Amphibia as described recently by Goodchild. The evidence that mammals regenerate new kidney tubules (p. 391) is one of the most important additions in this section.

There is no account of the growing body of results on biochemical aspects of regeneration and wound healing in mammals, or of the physiological and clinical fields of Cuthbertson, Seyle and others. These aspects surely must have advanced in Russia, also, and it seems unlikely that the book will greatly attract physicians (p. x) without some account of them.

There is a healthy intussusception of new facts about regeneration in other vertebrates and in many of the invertebrates and this is reflected in the large number of new illustrations. However, little of it is sufficiently novel to western readers to demand emphasis here. As in the case of the Amphibia and Mammalia some of the available work from outside Russia was overlooked, including experimental work. Some of that of Bröndsteds' group and of a number of French workers, on the lower invertebrates, might be mentioned. Among the general facts which emerge is the great extent to which regeneration recapitulates embryogenesis, but not in all animals.

In the section on physiological regeneration there are a number of new facts and some interesting extensions of the scope of the term. It is perhaps surprising that the authors should exclude replacement-turnover at the molecular level. The views of Pavlov quoted on p. 79 appear to encourage the more comprehensive view. At the other end of the scale it would be reasonable to regard reproduction as a physiological regeneration of losses among individuals, and so to forge another bond between the sections of the book. The tables and summaries in this section, as well as some of those elsewhere, tend to deal with rather minor matters.

Points of general theory are interspersed through the text and the main topics are reviewed and summarized in the last chapter. One at least, on the "histogenetic laws of the regenerative process", is more parochial and might with advantage have been placed at the end of the preceding chapter. That on environmental conditions has practical rather than philosophical significance. The authors repeatedly emphasize (e.g. p. 231) that environmental conditions have often been ignored in comparing results and in some instances it is possible that errors and confusion have resulted, but in general it seems that purely observational studies have aimed at consistent conditions, as nearly natural as possible. In experimental work, provided there is an adequate control for the one factor under test the background conditions need not be critically important. The importance of control experiments might have been further stressed. Cases where a mention of them would have been reassuring include Umanski & Kudokotsev's interesting experiments (p. 372) showing that parathormone stimulates regeneration in mammalian limbs and the experiments on avitaminosis A and D (p. 373).

The discussions are shrewdly critical and the theories of Russian authors are subjected to the same scrutiny as others. For instance the theories of Polezhayev (p. 315) and of Lepeshinskaia (p. 368) that cells may form *de novo* from non-cellular materials are not approved. The overriding sense of fairness of the authors,

however, results in many controversial problems being left unresolved and the reader in doubt of the authors' casting vote. Instances are the role of I-cells in coelenterate regeneration (pp. 171–175) and the effect of repeated regeneration (pp. 413, 415).

As severe critics the authors are cautious about propounding new theories of their own and the claim of the advertisement that "For the first time a full biological explanation of the problem is presented" should not be taken to mean a solution of the problem. Their theory of the "adaptation" of tissues to each other (p. 443) deserves amplification, if only in reply to the implication above that mutual "induction" or "organization" would still suffice. The new term "regenerative hypertrophy" which they suggest (p. 184) for the restoration of the size of a partially excised organ by growth elsewhere than at the cut surface, seems scarcely necessary. "Compensatory hypertrophy" has usually been taken to include this situation.

One of the most important philosophical questions discussed is that already mentioned: does regenerative power decline with the evolution of tissue differentiation, as Weismann contended? In further defence of his view may be stressed the invariable tendency to dedifferentiation as a prelude to growth during regeneration, in tissue culture, and in cancerous tissue. Again, notwithstanding exceptions mentioned (p. 423), in general regenerative power declines progressively during each lifetime. Certainly there are a number of exceptions to the general correlation but these do not invalidate it. The exceptions can often be related to a variable survival value of regenerative power in different animals and different organs. This correlation, also, the authors are inclined to deny, because it again is not absolute. In fact each will tend to depress the correlation coefficient of the other but this partial correlation does not rule out either as a major factor determining the incidence of regenerative potency.

Certainly there are cases where at present the potency cannot be clearly related to the degree of susceptibility to injury but this is usually due to ignorance of the latter. Positive evidence against the correlation is largely confined to Morgan's often quoted work on the hermit crab. It is possible to interpret Morgan's results in favour of the correlation rather than against it, and "laws" based on his interpretation (p. 412) seem unwise. The authors rightly point out that *all* the physiological functions of animals necessarily are adaptive, that regeneration must be a pristine adaptation and therefore that adaptiveness is too axiomatic to need stating, unless it carries the implicit antithesis that animals have also properties of non-adaptedness. This is not the only possible antithesis however, and the significant one here is that the *absence* of regenerative potency, in certain animals and organs, also is adaptive. In some short-lived animals, with large numbers, regeneration would be a wasted effort. The authors quote the great ability of enteropneusts to regenerate their head and poor ability to replace the tail as being non-adaptive but these tubicolous animals appear to expose the head far more than the tail and appear to conform to the generalization that sedentary animals regenerate best the head and actively errant types the tail.

Ideas which appear new and stimulating include: (p. xx) In paratomy functional isolation precedes and in architomy it follows anatomical isolation; (pp. xxiii, 424) Starvation causes regressive changes similar to dedifferentiation: conceivably this may account for the poor appetite of animals in the early stages of regeneration; (p. 110) Regeneration provides the most crucial evidence on the epigenesis-preformation controversy; (p. 356) The different tissues of one organ are more compatible morphogenetically than the same tissues from different organs.

Ideas usually considered important in other circles but given less emphasis in this book include Weiss' theory of tissue "modulations", i.e. minor changes of fate within a major group of tissues (p. 427). Another leading controversy deserving consideration is the question whether epidermal cells over the blastema sink in to provide internal tissues. On the more general question, the authors assess the evidence as being against metaplasia in the vertebrates but in favour in tunicates and some invertebrates. Here asexual reproduction does provide interesting evidence.

The views of Weiss and others on organ-specific inhibitory substances also deserved consideration already in 1957. The evidence mentioned above that all tissues of one organ have common compatibility properties seems to mollify the objection previously raised that it is necessary to envisage a specific inhibitor for every tissue of every organ. Lehmann's evidence of stage specific inhibitors is another important recent development; again the possibility that the epidermis mainly controls early stages and the deeper tissues later stages of regeneration perhaps merits attention. In the theoretical section the relation between regenerative ability and indeterminate growth might have been considered and also certain other correlations, such as body size.

The book shows careful attention to correct anatomy and systematics; lapses, apart from the spelling of generic names, are virtually limited to grouping the acarines with the myriapods (p. 256), using "coral polyps" for the Zoantharia in general (p. 185 etc.), showing *Campanularia* without hydrotheca (Fig. 55), and referring to "nephridia" in the Enteropneusta (p. 278) and to an ectodermal coelomic lining in *Harmathoe* (p. 229). Nusbaum's two "strains" of *Lineus ruber* (p. 206) have proved to be two species.

The term "spireme" (p. 6) for early prophase chromosomes is startling and "apodermis" (p. 262) is surely a mistake for "apodemes" ? Geneticists may object to the term "inheritability" applied to the ability to reproduce morphological abnormalities from one act of regeneration to another (p. 442). Conceivably branches of the fifth cranial nerve may prove to be more relevant than the olfactory for snout regeneration (p. 331).

The historical introductions to the various sections are a valuable feature. In some cases there may seem to be an over-emphasis on the work of Russian biologists but there may be a contrary tendency abroad and authors render a service by documenting this work fully. For this and for more general reasons it is a great pity that the literature list of the book is so short. This is not unreasonable in a student's text-book but for workers abroad it could have been a key to the

Russian literature. Certainly the text references are often dated, so that some could be traced in the abstracting journals but not all references are given, even in the text. There is no author index.

The authors would probably admit that even in the original edition a number of the figures are small and obscure and not always very informative. No doubt illustrations for this subject do not need excessive anatomical detail but there should be enough to provide a real supplement to the text. Fig. 139 has no landmarks at all. By an oversight Fig. 164 has been omitted from the English edition. There is an original and obscure error in the reference on p. 435 to Fig. 136.

In evaluating the work from the English edition the translator's contribution is important. He deserves high praise for the skill with which a technical subject with an extensive terminology has been translated from one difficult language to another. He has struck a nice balance between a literal translation and a free English style; he has reduced the number of short casual paragraphs and in other ways improved the tactical details of the work.

In some places satisfactory translation could have been ensured only with the additional help, with all the time this would entail, of a specialist on the subject matter. For instance it is a matter of great importance that "rasvitie" should usually be translated more literally as "development" than as "growth". Without specialist help inevitably some subtleties are lost and some obscurities become worse. Unless translation is freely in the technical idiom the phraseology appears naïve and then confidence in the authority of the text is unwarrantably undermined. "Organ remnant" for "stump", "posterior vent" for "cloaca", "protein degeneration" for "proteolysis", "intra-organic" for "inside the organ" are a few examples.

Examples of the magnitude to specialists of small errors of translation include the rendering of "yashcheritsa" as "pholidota" (pangolins) instead of "lizards" (pp. 135, 139), so implying that mammals readily regenerate tails! Again the translation "deer" instead of "antelope" (p. xv) makes bad biological sense of the shedding of horns. A biologist would not substitute "organic matter" for "organisms" (p. 454) or call annelid segments "fragments" (p. 238). Other errors which possibly should be mentioned, in fairness to the authors or to the reader, include "larvae" for "Larvacea" (p. 54), "Norman fishermen" for "Normandy fishermen" (p. 111), "regeneration from eight segments" for "regeneration from the eighth segment" (p. 232, Fig. 118), "marine" for "aquatic" Pulmonata (p. 248), "dorsal suckers" of Nudibranchs (p. 248), "regeneration throughout their entire extent" for "regeneration after complete extirpation" (p. 371) and "blind pouch" for "auditory vesicle" (p. 437). On p. 423 regeneration is said to be better in early than in later stages, an inversion of the authors' statement, and on p. 451 the bud of *Hydra* does not literally replace the parent's head, but only functionally and morphallactically. Difficulty with the translation is seen in the legends of Figs. 188 and 364 and in passages on pp. 318 (last paragraph), 339 (second paragraph), 345 (2 and 3b), 355 (top) and 362 (first paragraph).

Inevitably there are difficulties in translating back into latin script the names of non-Russian authors and in some cases error could be avoided only by prior

knowledge of the name; the translator therefore was not misled by two different spellings of "Huxley" but "Gertvig" (Hertwig), "Dzidzh" (Gidge) and others are understandable. Unfortunately without a full bibliography it may be difficult to correct some of these. Occasionally the translator is caught off guard e.g. in Della Balle (Valle) and Balett (Valette) and it is difficult to condone his use of the apostrophe in place of the silent Russian letters Ь and Ы, particularly in non-Russian names.

He is probably not responsible for asexual "propagation" rather than the zoologically preferable "reproduction" on the jacket and title-page, since the latter appears in the running title throughout. The classification, in the scheme of Fig. 72, of heteromorphs as quantitative aberrations, also may be the error of someone else. The original already had "incomplete regeneration" and "regeneration hypertrophy" as examples of qualitative aberrations so that this further change completed an inversion of the logical classification! Completion of the similar kind of scheme on p. 3 appears to have been overlooked, but the space in the text near the top of p. 231 does not imply any omission.

It is necessary to recognize the true value of the book through the technical drawbacks which inevitably surround a translation, and through whatever original shortcomings it may have. It remains the most valuable recent collection of facts, in English, both on the incidence of regenerative and asexual reproductive activity in animals, and on the general nature of regeneration.

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# BOOK REVIEWS

**Polysaccharides of Micro-organisms.** M. STACEY and S. A. BARKER. Oxford University Press, 1960. ix+228 pp., 30s.

THE polysaccharides synthesized by micro-organisms are of special interest to the carbohydrate chemist. Firstly, many of the polysaccharides occur in complexes with protein and phospholipid material so that their isolation, properties and functions have to be considered in relation to that of other cell constituents. Secondly, the component sugars often include monosaccharides which are not commonly found in plant or animal polysaccharides, e.g. the amino-sugar *D*-fucosamine, the 3, 6-dideoxy hexoses abequose, colitose, paratose and tyvelose, and heptoses, e.g. *L*-glycero-*D*-mannoheptose and *D*-glycero-*D*-galactoheptose. Thirdly, many of the polysaccharides or polysaccharide complexes show specific biological activities. These aspects have been particularly emphasized in the present volume, which provides a comprehensive and up-to-date survey of the chemistry of microbial polysaccharides.

The first five chapters serve as an introduction for non-specialists. These cover respectively carbohydrate nomenclature, monosaccharide components of polysaccharides, isolation and homogeneity of bacterial polysaccharides and their complexes, and finally, structural determination of polysaccharides. The latter chapter is not entirely satisfactory; many techniques are described, e.g. acid hydrolysis, periodate oxidation, infra-red spectrophotometry, and immunological reactions but enzymic methods of analysis (examples of which are given in later chapters) are not discussed. Some practical details of the methylation analysis of polysaccharides are included, but its purpose, advantages and limitations are not mentioned. The method of partial acid hydrolysis (linkage analysis) also merits further discussion.

The remaining seven chapters contain a systematic account of the polysaccharides of rickettsiae and viruses, of Gram-negative bacteria, of some Gram-positive bacteria, of the higher bacteria, of moulds, of yeasts and yeast-like fungi, and finally, of the protozoa. The book also contains two useful appendixes, the first tabulating the physical constants of approximately thirty monosaccharides which may be present in the polysaccharides of micro-organisms, and the second, summarizing recent reports of polysaccharides in micro-organisms, the majority of which were published in 1959. There is a separate bibliography for each chapter; this is a valuable feature of the book, although once or twice an unsatisfactory reference to "unpublished results" is given.

The information covering microbial polysaccharides was scattered throughout both the scientific and medical literature, and the authors are to be congratulated on their success in collecting and arranging the data. Tables giving the properties of *Aerobacter-Klebsiella* polysaccharides, the numerous *Salmonella* polysaccharides, and the various bacterial dextrans will be of particular interest.

Since most experimental work has been carried out on the pneumococcal polysaccharides, these are described in considerable detail. The accounts of the Type II, III, VIII and XIV polysaccharides, in particular, illustrate the successes which have been achieved in this field. The dextrans, bacterial levans and nigeran are also covered in detail. The main structural features of numerous bacterial polysaccharides, e.g. from *Acetobacter*, *Azotobacter*, *Neisseria*, are known but details of fine structure are not yet available; however, in other cases, there are many gaps in present knowledge both with regard to the composition of the polysaccharide and the nature of the glycosidic linkages. The experimental techniques in this type of analysis are often difficult, and it is evident that there is an unlimited number of problems for the carbohydrate chemist in microbiology.

A copy of this book should be available to all interested in the chemistry and biology of microbial polysaccharides and it is clearly an essential purchase, at a very modest price, for all research workers in this branch of biochemistry.

D. J. MANNERS

J. BRACHET: **The Biochemistry of Development.** Pergamon Press, London, 1960. 320 pp., 65s.

THE application of biochemical techniques and ideas to the study of embryos has a powerful appeal to the younger generation of biologists, especially in the United States. These systems that change rapidly according to a predictable pattern challenge the investigator with new dimensions for precise analysis: not only problems of the control of reaction rates within a cell, little enough explored, but of control of the orderly change of the mechanisms of control themselves. In such an attractive subject, lying at the border between two rather distinct disciplines, some means of orientating newcomers is badly needed. It is now 10 years since the last edition of Brachet's classic *Embryologie Chimique* and it no longer entirely suffices. Brachet's new and shorter book admirably fills the need. It is not at all a mere compilation of results, a means of orientation in existing knowledge (though plenty of existing knowledge is included). The orientation the reader receives is strongly biased towards the gaps in existing knowledge. It is a book by a man absorbed in research, written with all Brachet's robust common sense yet feeling for new horizons, to stimulate his present and above all his future colleagues.

The gaps in knowledge, all papering over ruthlessly torn away by Brachet, are fundamental. The central problem of the subject is of course how in chemical terms the cell-types differentiate. (Though Wilde has recently been producing mixed-up cells, differentiation is essentially based on cellular units.) This differentiation involves the appearance, in different localities of the early embryo, of cell lineages which have new structure, new responses to surroundings and new underlying metabolic patterns, and which replicate these characteristics in a rather stable way. The localization within the embryo of the various types of differentiation usually depends on mutual influences of the developing cells, and classical experimental embryology has been mostly concerned with these actions and interactions, notably in the analysis of induction. For many years the mode of this intercellular communication has seemed a sitting target for biochemistry. A responsive cell population can be relatively easily arranged as a test system for the action of substances which might be transmitters of the differentiating influence. But it is remarkable how frustrating the outcome of such investigations has tended to be, though naturally they have had useful by-products. The responding system answers with its characteristic process of differentiation, such as vegetalization in the echinoderm or neuralization in the amphibian, to far too many different substances. Cells turn out in fact to be complex systems with a life of their own, liable to give a stereotyped response to a variety of different stimuli.

Since cells in their responses are such self-contained organisms, extra emphasis is thrown on the need to know the internal chemical mechanisms of their differentiation. The study of what goes on inside developing cells is still largely in the preliminary phase of listing and quantitizing the occurrence of various components during development. Brachet is particularly good in demonstrating by example how this essential groundwork can be most effectively laid by drawing together results from a variety of techniques, from electron microscopy, serology, autoradiography and cytochemistry as well as the more usual biochemical methods. But the general impression left by much of this descriptive work, as Brachet is not slow to point out, is still of chaos, not only in hypotheses but even in experimental results. It is evident that single parameters, however fundamental they may seem and careful their measurement, are often subject to incomprehensible variations from region and species to species. Small wonder that the process of cell transformation itself

remains almost untouched by biochemistry. Since quantitative change in the enzyme make-up of a cell must be fundamental to differentiation, the spectacular current advances in knowledge of protein synthesis will ultimately open new vistas for embryology; but these advances as yet hardly touch the problems of change.

This may seem a depressing picture, but it is not so at all as Brachet presents it. It is an exciting picture of a subject ready for the establishment of its fundamentals, and when these fundamentals emerge it will be a major biological advance, comparable with the breakthrough in genetics. In its formative years the subject has been extraordinarily fortunate in having the guidance of Needham and Brachet, both with a great knowledge of this complicated and intransigent material, and a great faith in the power of biochemical analysis. It is to be hoped that their enthusiasm and breadth of interest will be a persistent influence when the subject comes to maturity.

M. ABERCROMBIE

M. FRANK MALLETTE, PAUL M. ALTHOUSE and CARL D. CLAGETT: **Biochemistry of Plants and Animals, an Introduction.** John Wiley, New York, 1960. 552 + xiii pp., $8.50.

THIS elementary textbook is designed for students in the various agricultural curriculums. It assumes only a basic background in organic and inorganic chemistry on the part of the reader. The subject coverage is very broad as implied by the title. The book is organized into three sections titled "General Biochemistry", "Plant Biochemistry" and "Animal Biochemistry". The classical topics of a biochemistry textbook are supplemented with special chapters on seed germination, growth regulation (in plants), nutrition and feeds. The broad subject coverage has been accomplished with the sacrifice of comprehensive detail in many cases. Exciting modern developments in such subjects as protein structure, protein synthesis, biochemical genetics and biochemical controls have been treated superficially or omitted completely. This textbook seems to be reasonably well written, and it should be adequate for a terminal course in biochemistry.

R. G. WOLFE

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# IRON BINDING BY VERTEBRATE BLOOD SERA*

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(*Received* 14 *September* 1960)

**Abstract**—(1) Specific iron-binding components were demonstrated in rat, chicken, turtle, frog and fish sera by using starch gel electrophoresis and radioactive iron$^{59}$.

(2) One iron-binding component was identified in each serum except turtle, which had two.

(3) The serum iron values and unsaturated iron-binding capacities were determined for rat, chicken, turtle and frog sera.

(4) The serum iron-binding components were similar to the serum components previously identified as inhibitors of lipid peroxide formation.

(5) Although the mechanism of inhibition of lipid peroxide formation in all sera appears to be iron-binding, there exist specific chemical differences in the chelates formed.

## INTRODUCTION

MAMMALIAN blood inhibits the autoxidation of many labile compounds including ascorbic acid (Barron *et al.*, 1936) and dihydroxyphenylalanine (Monder *et al.*, 1958). Blood sera from a wide variety of vertebrates also inhibit peroxide formation in aerobically incubated rat brain homogenates (Barber, 1961). The mechanism of inhibition of peroxide formation in each instance appears to be the binding of iron since:

(1) Iron catalyses and chelating agents inhibit lipid peroxide formation (Barber & Wilbur, 1959).

(2) The rat serum inhibitor migrates electrophoretically in the beta globulin region, and this region possesses the iron-binding protein in mammalian serum (Allison, 1959).

(3) The amount of iron necessary for the reversal of rat serum inhibition is approximately that which has been reported for rat serum unsaturated iron-binding capacity (Kaldor, 1954).

The mechanism of inhibition of lipid peroxide formation by sera from non-mammalian vertebrates also appeared to involve chelation (Barber, 1961). This mechanism was suggested on the basis of iron reversal studies. No direct evidence for this mechanism could be offered, however, since few data were available on the unsaturated iron-binding capacities of these sera and nothing was known about the electrophoretic behavior of their iron-binding components. This study reports

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† Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

the results on these two phases of vertebrate serum iron. The mechanism of inhibition is elucidated by direct comparison of serum unsaturated iron-binding capacity (UIBC) and the degree of inhibition of lipid peroxide formation by serum.

## MATERIALS AND METHODS

Electrophoresis was carried out on starch gels according to Smithies (1955). $Iron^{59}$ (0·1 $\mu$c) was added to 0·5 ml of serum (20 mg protein/ml), and 0·1 ml of this mixture was applied to a filter strip and inserted into the gel. The gels were made up in 0·02 M phosphate buffer pH 7·0. The electrode solution, also pH 7·0, was brought to 0·2 M with NaCl. Electrophoresis was carried out at 9 V/cm at 4°C for 12 hr. The gels were then stained with buffalo black after being split longitudinally. Iron localization was determined by scintillation counting after cutting the unstained half into 3 mm segments. Protein was localized by scanning stained gels directly with a Photovolt densitometer.

Determinations of serum iron, unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC) and per cent saturation were carried out on 2 ml of serum according to the method of Schade *et al.* (1954).

Blood was collected from adult male rats (Sprague–Dawley) and 6 in. turtles (*Pseudemys elegans*) by exsanguination after stunning and decapitation. 6 in. bull frogs (*Rana catesbiana*) and adult yellowtail fish (*Seriola dorsalis*) were bled directly from the heart, and adult chickens (Rhode Islands ♂ vs. New Hampshire ♀) were bled from wing veins. Serum was collected from clots after centrifuging for 15 min at 10,000 *g*.

Studies on the inhibition of lipid peroxide formation were carried out using rat brain homogenates as the test system (Barber, 1961). Brains were homogenized in cold buffer (100 ml of 0·15 M NaCl, 4 ml of 0·15 M KCl and 10 ml of 0·1 M phosphate buffer at the pH indicated) and diluted 1 : 15 (w/v) with the appropriate buffer. Final pH adjustment was made with HCl. 2 ml of this homogenate was incubated with 2 ml of serum (adjusted to the indicated pH and concentration) in 20 ml beakers. Controls received 2 ml of buffer. Beakers were incubated for 90 min at 37°C in a Dubnoff metabolic shaker oscillating at 100 cycles/min. Lipid peroxides were determined with thiobarbituric acid (TBA) according to Barber & Wilbur (1959).

## RESULTS

Blood serum proteins from rat, chicken, turtle, frog and fish were separated by starch gel electrophoresis (Fig. 1). Arrows designate the localization of the radioactive ion. The total number of components separated, as well as the electrophoretic mobility of the serum iron-binding components, varied greatly among sera from different species.

Rat serum separated into at least five, possibly six, distinct protein components. The beta globulin region separated into two components, and all of the $iron^{59}$ was localized in the faster moving ($B_1$) component (Figs. 1 and 2). Such localization would be suspected from previous work on mammalian transferrin (Allison, 1959).

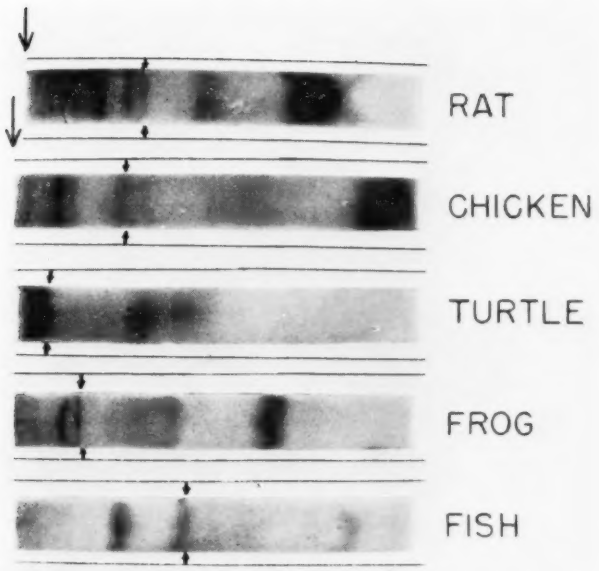


FIG. 1. Electrophoresis of vertebrate blood serum on starch gel. Separations carried out for 12 hr at 4° C using 9 V/cm. Gels stained with buffalo black. Short arrows indicate localization of iron$^{59}$. Long arrows indicate starting boundary at extreme left of gel. Cathodal portion of gels not photographed.

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The elevated activity at section O represents that portion of the isotope which did not leave the starting boundary. A greater or lesser amount of residual activity was noted on the starting boundary of all samples examined (Fig. 3). Recovery of iron applied to the starch gel was examined in the case of rat serum and was found to be 91 per cent.

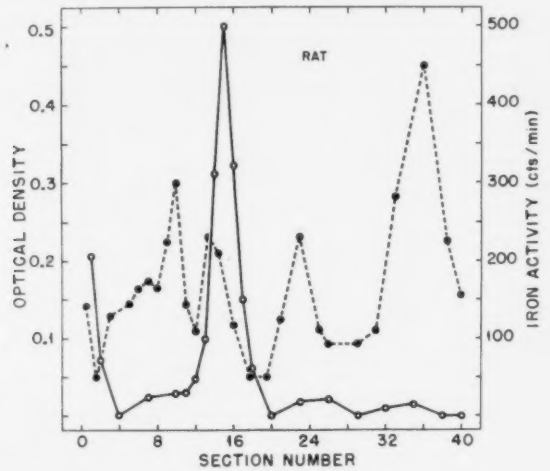


FIG. 2. Iron binding in rat serum. Electrophoretic characteristics as in Fig. 1. Iron activity determined on 3 mm sections of the gel using scintillation counting. Optical density determined directly on the gels using a Photovolt densitometer. Iron activity (○); Optical density (●).

Chicken serum separated into the greatest number of protein components of all sera tested (Fig. 1). In chicken, as in rat, all iron[59] was localized in a single protein component (Fig. 3). The migration of the iron-binding component in chicken was similar to that of rat and appeared to be associated with the beta globulin fraction.

Turtle serum was different from all other sera in that two iron-binding components were present (Fig. 3). These were associated with two distinct proteins that appeared to constitute a high percentage of the total serum protein. One of these peaks migrated towards the anode, and one migrated towards the cathode (not shown in Fig. 1) at pH 7·0. Neither component migrated any appreciable distance from the starting boundary. The electrophoretic differences indicate major physico-chemical differences between these proteins and proteins of similar function in the other vertebrate sera. Two minor components migrated toward the anode and neither was associated with iron binding.

The iron-binding proteins in frog and fish sera resembled electrophoretically iron-binding proteins of rat and chicken sera (Fig. 3). A single component carried all of the iron[59] activity in both animals. The intermediate mobilities of these components in frog and fish suggested that the proteins were electrophoretically similar to mammalian beta globulin.

Serum iron, UIBC, TIBC and per cent saturation were determined on rat, chicken, turtle and frog sera (Table 1). The UIBC of rat, turtle and frog were similar, whereas that of chicken was considerably higher. Chicken also possessed more serum iron and had a TIBC approximately twice that of the other sera

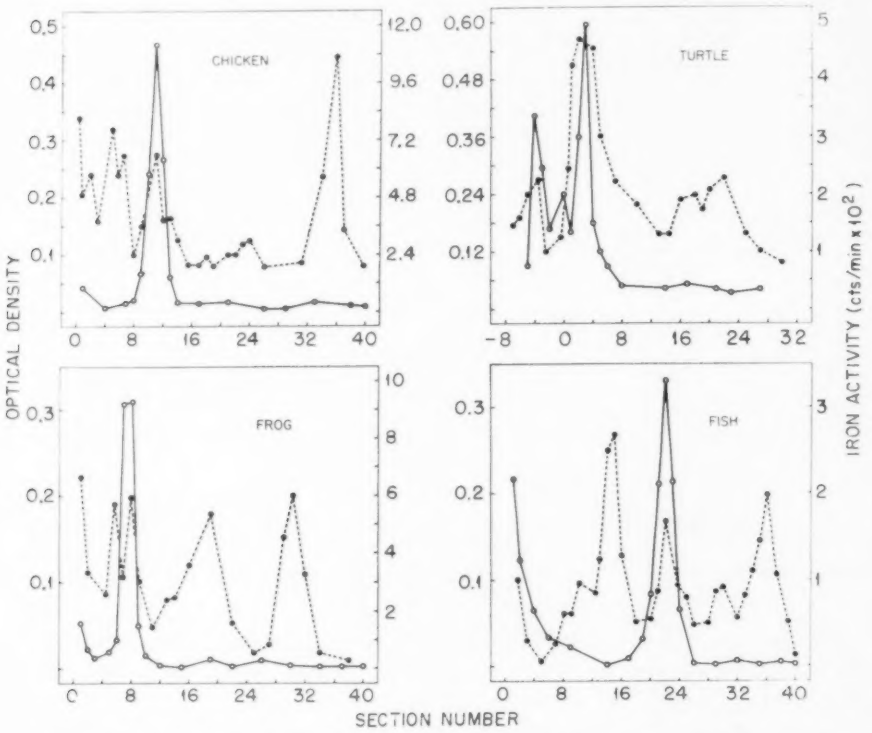


FIG. 3. Iron binding in chicken, turtle, frog and fish blood. Electrophoretic separations and determinations of iron activity as in Figs. 1 and 2. Iron activity (○); Optical density (●).

examined. The low values for serum iron in both the turtle and frog resulted in lower values of per cent saturation than were found in the rat in spite of the fact that all three possessed similar UIBC values. It is this latter value which is of obvious significance in the inhibitory activities of the blood associated with iron chelation. Considerable variation was noted in both the serum iron values as well as the UIBC values for animals of the same species.

The antioxidant activity of blood sera, as measured by inhibition of lipid peroxide formation in rat brain homogenates, has been recently reported (Barber, 1961). The serum inhibitors of lipid peroxide formation were identified as specific proteins and their electrophoretic migration compares favorably with the electrophoretic migration of the serum iron-binding proteins reported in the present study.

Also, the amount of iron needed for iron reversal of the inhibition of lipid peroxide formation compares favorably with the UIBC values obtained in the present study. The mechanism of inhibition of lipid peroxide formation, therefore, appears to be

TABLE 1—VERTEBRATE SERUM IRON AND IRON-BINDING CAPACITIES
Values are in micrograms Fe/100 ml serum

| | Rat | Chicken | Turtle | Frog |
|---|---|---|---|---|
| Serum iron | 147·0 [4]* (135·0–157·5) | 366·9 [4] (317·5–445·0) | 87·6 [4] (45·0–120·0) | 98·3 [3] (57·5–130·0) |
| UIBC | 287·5 [4] (230·0–355·0) | 417·5 [4] (377·5–500·0) | 276·0 [5] (207·5–367·5) | 285·5 [5] (245·0–307·5) |
| TIBC | 434·5 (382·5–512·5) | 784·4 (715·0–845·0) | 363·6 (262·5–458·0) | 383·8 (337·5–410·0) |
| % Saturation | 34·2 (30·8–39·9) | 46·7 (40·8–54·1) | 23·8 (15·5–36·7) | 24·4 (17·1–31·7) |

* The number in square brackets represents the number of samples examined.

iron chelation. The species differences in the electrophoretic migrations of these functional proteins suggested that physico-chemical differences existed between them. To further test this difference, the response of the inhibitors to various pH levels was determined (Table 2). All sera inhibited at pH 7·0. At pH 5·2, however,

TABLE 2—EFFECT OF pH ON SERUM INHIBITION OF LIPID PEROXIDE FORMATION
(Activity tested at 2 mg of protein)

| pH | Percentage inhibition of peroxide formation | | | |
|---|---|---|---|---|
| | Rat | Chicken | Turtle | Frog |
| 7·0 | 89 | 90 | 100 | 84 |
| 6·0 | 63 | 89 | 99 | 45 |
| 5·2 | 2 | 48 | 45 | 0 |

the inhibitory effect of frog and rat sera was abolished, while considerable activity persisted in both chicken and turtle sera at this pH. At pH 6·0, however, the inhibition by frog and rat sera was intermediate, whereas that of turtle and chicken sera was still near maximum.

## DISCUSSION

Lipid peroxide formation in rat tissue homogenates, or their fractions, is an iron-catalysed non-enzymatic oxidation that is inhibited by chelating agents (Barber & Wilber, 1959; Thiele & Huff, 1960; Tappel & Zalkin, 1960). A variety of tissues inhibit this reaction including rat intestinal mucosa (Barber & Wilbur,

1959) and vertebrate blood sera (Barber, 1961). The mechanism of inhibition by intestinal mucosa is enzymatic involving a phospholipase (Ottolenghi & Bernheim, 1960). In contrast, the mechanism of inhibition in the blood and sera involves the chelation of iron. This is indicated by the following:

(1) Electrophoretic migration of these inhibitors is similar to the migration of the iron-binding components.

(2) The inhibition can be reversed by amounts of iron which correspond to the unsaturated iron-binding capacities of these sera (Barber, 1961).

All sera examined possessed specific iron-binding components. These serum components varied in electrophoretic mobility, indicating physico-chemical differences. In mammals the iron-binding protein is a beta globulin (Allison, 1959; Laurell, 1960). Electrophoretic mobilities of the iron-binding components in chicken, frog and fish indicate that the protein may be a beta globulin similar to that of the mammalian serum. Turtle serum, however, possesses two iron-binding components, neither of which electrophoretically resembles the beta globulins of the mammalian serum. One migrates anodally at pH 7·0 whereas the second migrates cathodally; neither migrates appreciably from the starting boundary. The serum iron-binding components are similar to the major serum components previously identified as inhibitors of lipid peroxide formation (Barber, 1961). The minor inhibitory components of chicken and frog sera, however, do not appear associated with iron binding. In the case of turtle serum, both inhibitors appear associated with iron binding.

Studies of pH effects indicate chemical differences in various iron-protein complexes. The chemical nature of human transferrin has been studied more extensively than the iron-binding proteins of other sera (Laurell, 1960). It releases its iron at pH 5·2 especially in the presence of 0·2 M phosphate buffer (Schade *et al.*, 1954). Rat and frog sera do not inhibit lipid peroxide formation at pH 5·2, indicating that the mechanism of inhibition is reversible iron binding. On the other hand, chicken and turtle sera continue to inhibit peroxide formation at pH 5·2, albeit at a reduced level. Two explanations can be offered for these observations. The first, and more probable, is that a limited amount of iron binding occurs in chicken and turtle sera at pH 5·2, thereby suggesting the different chemical nature of these inhibitors. The second is that inhibition at pH 5·2 occurs by some mechanism other than iron binding and that this mechanism is only slightly changed at this pH. Different affinities between proteins and iron can be suspected on existing information. Conalbumin, for example, forms stable iron complexes at pH values down to 5·5 and continues to bind down to pH values of 4·0 (Warner & Weber, 1953). Rat serum, on the other hand, appears to release its bound iron at pH values of 6·0 in the presence of 0·2 M phosphate buffer (Schade *et al.*, 1954). If the chicken and turtle serum iron-binding proteins are similar to conalbumin, inhibition would be expected at lower pH levels. Studies on the chemical nature of the iron-protein complex in vertebrate sera have been initiated in this laboratory.

Species differences in serum proteins have been examined electrophoretically (Deutsch & Goodloe, 1945; Deutsch & McShan, 1949; Dessauer & Fox, 1956;

Drilhon, 1959; Gleason & Friedberg, 1953; Moore, 1945). These differences suggest different ionic properties of the serum proteins from various species. Since iron binding by serum proteins is also dependent on the ionic properties of the proteins, it is natural to assume that differences in iron binding should exist in various species. If certain animals do possess serum proteins capable of iron binding at pH 5·0, the accepted methods for determining serum iron may not be applicable for these animals. This characteristic, however, would not interfere with present methods for determining UIBC, and it is this characteristic upon which the inhibitory activity of serum on peroxide formation is based. It is important to note that the Greek letters applied originally to peaks in human electrophoretic patterns are operational designations and do not apply without reservation to other species. For example, the gamma or slowest moving peak in fish sera may not contain antibodies.

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Serum iron and iron-binding capacity vary under various physiological and pathological conditions. Variations in man have been detailed by Laurell (1947) and Rath & Finch (1949). In rats, sex and age variations have been noted (Kaldor, 1954). Egg laying in chickens has a pronounced effect on the serum iron levels (Ramsey & Campbell, 1954).

Increases in the levels of transferrin in sera in mice with transplantable hepatomas have been reported, but no information regarding the iron-binding capacity of these sera has been presented (Clausen *et al.*, 1960). Whether an increase in transferrin accompanies the increase in serum iron and whether increase in serum iron accompanies the increase in the level of iron-binding proteins is unknown. The physiological systems of Ramsey & Campbell (1954) and of Clausen *et al.* (1960) would be applicable to investigate this problem. The variations in serum inhibitory activity among individuals undoubtedly reflect the variations in UIBC.

Transferrin has been linked to the transport of iron (Ramsey & Campbell, 1954; Charley *et al.*, 1960). Nothing is known, however, about the iron-transporting function of serum iron-binding components in the lower vertebrates. Whether the chemical differences in the iron-binding proteins of these lower animals have any significance in iron transport is also unknown. These proteins do serve an important antioxidant function by their capacity to bind metal catalysts for autoxidation reactions. Which of these functions is primary for the protein might become understandable when more data are available regarding the iron-transport mechanism and the antioxidant activity of a wide variety of animals covering a significant portion of the evolutionary scale.

*Acknowledgement*—We are indebted to Dr. Norman G. Anderson, Biology Division, Oak Ridge National Laboratory, for helpful suggestions and criticisms regarding this work.

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# "RINGER" SOLUTIONS AND SOME NOTES ON THE PHYSIOLOGICAL BASIS OF THEIR IONIC COMPOSITION

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(*Received* 30 *September* 1960)

**Abstract**—(1) An outline is given of some of the biologically important properties of inorganic ions and of the part played by different ion species in the metabolism of cells.

(2) The composition of about 100 physiological salines for use with fish, amphibia, mammals, birds, annelids, crustacea, insects and molluscs is given together with analyses of the blood composition of some of the species of animals most frequently used in physiological experiments.

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## INTRODUCTION

PHYSIOLOGICAL salines have been described by Willmer (1954) as solutions of the necessary ionic concentration and osmotic pressure to allow cells to survive in them without damage. Regrettably, though not surprisingly, no simple solution can substitute for haemolymph over an extended period. In his classical study Ringer (1883a) showed that a bathing solution in which the ratios of the constituent ions are grossly incorrect results in the rapid death of a tissue, but one where the different ions are present in the correct balance will prolong the life of an isolated organ for some hours or days. An isolated frog's heart ceased to beat and became insensitive to stimulation some 20–30 min after transfer to NaCl isotonic with the blood: but addition of $CaCl_2$ to the solution resulted in the resumption of a spontaneous, though abnormal, beat. The inclusion of KCl neutralized the excessive contracture in the NaCl–$CaCl_2$ mixture and the heart beat was then normal for some hours. Buffering the medium with $NaHCO_3$ further prolonged the activity of the heart (Ringer, 1883b).

It is usually considered that a physiological saline should have a composition such that the metabolic behaviour of isolated cells and tissues is similar to that in the body of the intact animal; but, in a study of the "practical philosophy" of "Ringer" solutions, Beament (1960) points out that it is erroneous to imagine that it is possible to reproduce *in vitro* the behaviour and properties of a tissue which *in vivo* forms only a part of an integrated unit. Results obtained *in vitro* must therefore be applied with caution to the more fundamental problem of the behaviour of cells *in vivo*. Similarly the ionic requirements of cells should not necessarily be regarded as being similar to the concentrations present in the bulk of a satisfactory physiological saline. Many free charges are present on the surface of biological membranes and the ionic concentrations immediately in contact with cells may therefore be different from those in the bathing medium as a whole. However, the relative ion

concentrations at the membrane level are maintained by virtue of those in the bulk of the bathing medium, and it is thus the composition of the latter alone which has to be considered in the development of a saline. In life, most animal tissues are bathed by blood or an ultrafiltrate of blood and the most obvious composition for a physiological saline would seem to be one based on analyses of the blood composition. Though some of the most satisfactory salines are so based, allowance may have to be made in some cases for the absence of protein. The polyvalent proteins present in blood may be expected to modify the charge, and hence the ion concentrations, at the cell–medium interface. Solutions lacking proteins may thus have to have ion concentrations differing from those in blood if the concentrations at the interface are to be maintained at the appropriate level.

Adequate analyses of the haemolymph are not available for many species, however, and comparatively few of the physiological salines at present in use are based on the haemolymph composition, the ionic concentrations of most having been arrived at empirically. In order to assess the potential value of many of these salines it is therefore desirable that the general properties of different ion species and of cells in their ionic environment should be understood. To this end this paper has been divided into four main sections: (1) the biologically important properties and functions of ions; (2) special functions of the main ion species in cell metabolism; (3) lists of some of the physiological salines at present in use for the isolated tissues of vertebrates, annelids, crustacea, insects and molluscs with notes on the special features of the ionic metabolism and blood composition of these groups; and (4) a short discussion of the essential requirements of a physiological saline and some elementary points of procedure in their preparation.

Readers interested only in a general purpose saline for short-term experiments are advised to turn directly to the section on the appropriate animal. In the event of the saline selected proving inadequate some clues to the cause of failure may perhaps be found by reading Section 2. Section 1 is to be regarded as background information of theoretical importance only in the present state of our knowledge of the function of ions in protein metabolism.

More detailed accounts of ionic metabolism will be found in Bayliss (1959), Ussing (1960) and Fruton & Simmonds (1958).

## 1. GENERAL PROPERTIES AND FUNCTIONS OF IONS

The biologically important properties of ions are those which depend upon the atomic number, valency and tendency to form complexes with water and organic molecules. On these basic characters depend such diverse ion functions as (1) the stabilization of proteins in solution and regulation of their colloidal properties, (2) the activation of enzyme systems, (3) the development of electrical excitability, (4) the dynamic maintenance of isotonicity between cells and body fluids, and (5) the control of the permeability of membranes.

(1) Proteins are large molecules with many free positive and negative groups. Electrostatic interaction may occur either between groups of opposite charge or

with water. In the case of albumins the tendency for association of the charged groups with water is greater than that with opposite charges on neighbouring protein molecules. Albumins are therefore soluble in pure water. In globulins the protein–protein interaction is the more powerful and this class of proteins is insoluble in pure water. The addition of salts decreases the tendency for protein–protein interaction in globulins rendering the protein soluble, and, in addition, further stabilizes albumins in solution under the influence of unfavourable temperatures and pH.

Strong electrolytes in high concentrations, as a result of their greater ability to adsorb water, reverse the effect and tend to precipitate proteins. Efficiency in precipitating proteins and in affecting their other colloidal properties varies from one ion species to another as it depends on a function of atomic number and valency. As an illustration of the differences in effectiveness of different ion species in affecting the colloidal property of proteins a series is given below showing the decreasing order of efficiency of some ions in precipitating proteins.

$$Al > Ba > Sr > Ca > Mg > H > Cs > K > Na > Li.$$

(2) Little is yet known of the precise mode of action of ions in the activation of enzymes. In addition to the generalized effects which mono and multivalent ions have on the colloidal properties and hence stereochemistry of proteins, certain ions have more specific functions in the activation of enzymes. Possible modes of action of the ions on activation are discussed by Dixon & Webb (1958) and these may be summarized as follows: (a) the ion may form an integral part of the active centre of the enzyme, (b) the ion may link enzyme and substrate, (c) the equilibrium constant of the reaction catalysed may be shifted in the presence of a specific ion, (d) polyvalent ions may act by reducing the surface charge on the protein molecule. Indirect activation by ions may involve (e) combination with, and removal of, inactivating substances and (f) increasing the availability of activating substances by causing their release from other combinations.

(3) Active transport accounts principally for the unequal distribution of certain ion species across cell membranes. By virtue of this unequal distribution diffusion potentials are established, the sign and magnitude of which depend on the relative permeability of the membrane to the different ions present and on the ratio of the ionic concentrations.

The net charge on an ion tends to attract water dipoles and one of the factors of importance in determining the rate of diffusion of an ion through small pores in a membrane is its size when hydrated. The size of the hydrated ion is governed by the valency and atomic number, the attraction for water being increased with the valency and decreased as the number of electron shells is raised. The relative order of size of some common ions when hydrated is:

$$Al^{+++} > Mg^{++} > Ca^{++} > H^{+} > Li^{+} > Na^{+} > K^{+} = Cl^{-}.$$

The two smallest, K, and to a lesser extent Cl, are the principal ions involved in the expression of the resting potentials across cell membranes.

(4) Much of the osmotic activity of cells is provided by particles too large to diffuse out across the cell membrane. As cell membranes are permeable to small ions it might be expected that a simple Donnan equilibrium would be set up. If this were so, however, a net excess of particles would be present on the inside of the cell membrane and osmotic uptake of water would occur. In fact the osmotic integrity of cells is believed to be maintained as a result of the activity of a metabolic 'pump'. This 'pump' selectivity extrudes Na from cells thus setting up a double Donnan equilibrium, since the extrusion of Na has the same effect as if there were indiffusible ions on both sides of the membrane. There is thus no tendency for water to move into the cell if it is metabolizing in a normal manner.

(5) In some respects a parallel may be drawn between the action of mono- and di-valent ions and ion-exchange resins and on the permeability of cell membranes. The presence of free monovalent ions in a resin increases the osmotic pressure, and water uptake occurs until further swelling is prevented by the cross-linkages. Divalent ions are bound preferentially and, as they are less osmotically active within the resin, less water uptake occurs than in the presence of mono-valent ions (BDH Handbook). Similarly Osterhout (1956) has found that the presence of Ca decreases the water content of lipid membranes, whilst the water content of other biological gels is also regulated by the associated ions (ref. in Robinson, 1960). If the hydration of membranes is governed by the divalent ion content then the size of pores present might also be expected to vary to some extent as the membrane swells and shrinks. The permeability to ions and water might then in turn be affected.

A brief outline of some of the biological activities of the main ion species which may be of importance in the preparation of salines is given below. For more detailed accounts see Bayliss (1959), Ussing (1960) and Fruton & Simmonds (1958). Manery (1954) discusses the ionic relationships of various tissues.

## 2. SPECIFIC ION FUNCTION IN CELL METABOLISM

### *Potassium*

Potassium is the principal cation of all cells and its three main functions are associated with (1) contribution to cell O.P., (2) the potential across cell membranes and (3) activation of enzymes.

(1) It has been suggested that within cells K may be bound to organic components; but in muscles most of it must be in an ionized form if the cell is to be in osmotic equilibrium with the haemolymph. Furthermore Hodgkin & Keynes (1953) have shown that most of the intracellular K in nerve migrates under the influence of an electric field and is therefore presumably ionized. In some cells

phases are present, the K of which exchanges at different rates with the extracellular K (Harris & Steinbach, 1956). Many enzyme systems are activated by K, and since activation is presumed to involve an intimate association between ion, enzyme or substrate, the "binding" of a small proportion of the total cell K is to be expected.

(2) The resting potential, $E$, of muscle and nerve cells tends towards that given by the Nernst equation for the equilibrium potential for potassium:

$$E = \frac{RT}{F} \ln \frac{K_i}{K_0}$$

where $R$ is the gas constant, $T$ the absolute temperature, $K_i$ the intracellular potassium concentration, $K_0$ the extracellular concentration, $F$ is the Faraday (cf. Conway, 1957).

Changing the extracellular K concentration results in changes in membrane potential and also in the Cl distribution as this tends towards a Donnan equilibrium position such that

$$\frac{K_i}{K_0} = \frac{Cl_0}{Cl_i}.$$

The lowering of extracellular K below a critical value decreases the rate of extrusion of Na from cells and it is presumed that the extrusion of sodium depends on an active pump which exchanges Na for K.

(3) K activates many enzyme systems including pyruvic phospherase, fructokinase, glutathione synthesis and B-galactosidase and is an inhibitor of crab nerve ATPase and urease (cf. Ussing, 1960).

*Sodium*

Except in some phytophagous insects, Na forms the bulk of the cationic component of extracellular fluids. The Na of salines may be temporarily replaced in part or *in toto* by substances such as disaccharide sugars (Deyrup, 1953) or choline without disturbance of the osmotic balance of cells, and it was for long thought that the function of Na was the passive maintenance of extracellular osmotic pressure. However, depolarization of the cell membrane of the electrically excitable tissues, nerve and muscle, is intimately associated with a marked increase in the permeability to Na and the various phases of action potentials have been interpreted in terms of the movements of Na and K. Consequently replacement of the Na in the extracellular fluid, though not affecting the osmotic properties, would be expected to result in a loss of excitability in nerves and muscles. A loss of excitability in salines where choline replaces Na has been observed in vertebrate nerves and muscles (ref. in Hodgkin, 1951) and in squid nerves (Hodgkin & Katz, 1949). Vertebrate smooth muscle and *Carcinus* muscle are less dependent on the Na level, smooth muscle of the guinea-pig gut retaining its excitability until the Na level is reduced below 10 mM/l (Holman, 1957). In *Carcinus* muscle choline can

substitute for Na in the production of an action potential (Fatt & Katz, 1953); but even in this case the muscle soon dies in choline rich solutions, presumably because penetrating choline cannot be actively extruded from the fibre as can Na. The resting potential of excitable tissues, although not completely insensitive to the Na level, is not grossly affected by changes in the Na concentration of the extracellular fluid (Hodgkin & Katz, 1949). A decrease in the extracellular K level leads to the exchange of some intracellular K for Na, or H (Darrow & Hellerstein, 1958), whilst, conversely, a small rise in the extracellular K favours the extrusion of Na from cells. A rise in the Na level within cells is likely to have a deleterious effect, as Na is an inhibitor of some enzymes and is less effective than K in activating others. Entry of Na into the cell may not have an immediate effect, however, as specialized regions of the cytoplasm, such as mitochondria, may actively maintain their own internal ion concentration (Davies, 1954; Gamble, 1957). Glycolysis is inhibited by Na (Clarke & McLeod, 1954); but on the other hand Na appears to be an essential activator for the transfer of glucose from the gut lumen in toads (Czáky & Thale, 1960). A discussion of the effect of Na on other enzyme systems will be found in Ussing (1960).



*Calcium*

It is generally believed that Ca lowers the permeability of cell membranes to both water and ions (Heilbrunn, 1952; Robertson, 1941). This effect is of special importance in the metabolism of excitable tissues. Muscles in a Ca-deficient medium show spontaneous activity and unstable resting potentials. Excitability is soon lost. Recent evidence suggests that this increase in excitability in low Ca depends on an increase in sodium conductance. It is possible that during the action potential Na is carried into the cell by a carrier mechanism and that in the resting cell this carrier is blocked by Ca (Hodgkin, 1958). Similarly the removal of Ca from the medium abolishes the action potential in *Carcinus* (Fatt & Katz, 1953), whilst a rise in Ca increases the magnitude of the resting and action potentials in the stick-insect *Carausius* (Wood, 1957). Ca is also specially associated with the shortening of the contractile element in muscle (Bailey, 1942) and like Mg can initiate the contraction of muscles (Heilbrunn, 1952). Ca activates myosin ATPase; but according to Boyer *et al.* (1943) it antagonizes the K activation of pyrophosphatase. The chelation of Ca onto proteins plays a part in the gel–sol relationships of protoplasm and also in the maintenance of intercellular cement (Höber, 1945; Robertson, 1941). In the absence of Ca, cells tend to separate, an action which is aggravated by high phosphate levels. This fact has been utilized in the culture of epithelial cells, a high phosphate and low Ca then proving advantageous (Willmer, 1954).

Ca antagonizes Mg in several reactions. Mg decreases the release of ACh at nerve endings whilst Ca increases the release (Hoyle, 1955; Hutter & Kostial, 1954).

In muscle, Ca is present in about the same concentration as in blood (Harris, 1956); but as a divalent ion it would tend to be more readily chelated onto proteins than Na and K. Harris (1956) suggests that both Ca and Mg are to a considerable extent combined in a non-ionized form. Similarly, in the haemolymph, Willis &

Sunderman (1952) have found that about 50 per cent of both Ca and Mg are not freely ionized. This point is of considerable importance in the preparation of salines since vertebrate neuro-muscular junctions are extremely sensitive to quite small changes in the level of these ions (Castillo & Enbaek, 1954).

The swelling of isolated vertebrate nerves and liver slices is aggravated if Ca is absent from the bathing medium; but both water uptake and K loss are diminished by raising the K level in the bathing fluid to 10 mM/l and the Ca to 2 mM/l [Aebi (1952) quoted by Harris (1956)].

For further discussion of the functions of Ca see Höber (1945), Heilbrunn (1952), Clark (1958), Hodgkin (1958), Robertson (1941) and Frankenhaeuser & Hodgkin (1957).

*Magnesium*

In many reactions Mg behaves in a similar manner to Ca; but in other cases the action of Ca is antagonized. Like Ca, Mg decreases the permeability of cells and whole animals (McCutcheon & Lucke, 1928; Robertson, 1941), and it is also effective in maintaining intercellular cement. In this latter respect it is usually less effective than Ca, but Gray (1926) found that the intercellular matrix of *Mytilus* is maintained better by Mg than by Ca.

Mg has a depressing effect on contractile tissues and its absence from the bathing medium results in hypersensitivity. It occurs in high concentration in muscle, up to 35 mM/l, but higher concentrations are inhibitory if K is thereby displaced. Mg activates a number of enzyme systems including crab ATPase, pyruvic phosphatase and fructokinase (cf. Ussing, 1960). In plasma between 50 and 87 per cent of the Mg may be bound to organic components (McLean & Hastings, 1935; Willis & Sunderman, 1952). Antagonistic reactions of Mg and Ca are discussed later.

*Phosphate, bicarbonate and chloride*

The pH of the extracellular fluid is of critical importance to the normal functioning of cells and one of the principal actions of $HCO_3$ and $HPO_4$ is that of their contribution to the provision of adequate buffering capacity in the blood. More specific functions are less well known than those of the alkali metal ions but Fenn & Cobb (1934) and Boyle & Conway (1941) have found that higher concentrations of K are required in the medium to maintain the cellular K level of frog muscle if $HPO_4$ is omitted from the saline. Within cells the transfer of energy is intimately related to $HPO_4$ metabolism. High concentrations of $HPO_4$ in bathing solutions are detrimental if Ca is precipitated.

Lazer (1942) has shown that if $HCO_3$ is not present in the saline bathing vertebrate tissue slices they soon lose their reactivity to substrates. Conversely the presence of small amounts of $CO_2$ ensures the stabilization of $O_2$ consumption. Kerkut & Laverack (1957) have demonstrated a somewhat similar effect of $HCO_3$ on the respiration of an invertebrate, the respiratory rate of *Helix* tissues being markedly increased in a saline containing $HCO_3$. Bicarbonate is involved in some

exchanges of Cl between cells and bathing fluid (as in the well-known "Hamburger shift") and also probably in the active transport of Cl. Krogh (1939) has shown that crustacea can actively take up Cl from $NH_4Cl$ and $CaCl_2$ without the cation, electrical neutrality being maintained by $HCO_3$ loss from the body. Carey & Conway (1954) find that the presence of bicarbonate in saline favours Na extrusion from muscle, and Creese (1949) finds that muscle performance and retention of K by fibres is favoured by the presence of $HCO_3$ in the bathing medium. Creese (1952) also finds that isolated rat diaphragm muscle loses K in $HCO_3$ free saline but regains it on the addition of $HCO_3$.

Chloride is the principal anion of the extracellular fluid, yet little is known of its biological functions. Together with K, Cl plays a part in the development of the resting potential of frog muscle. The permeability of the membrane to Cl is greater than that to K in a resting fibre, but under appropriate conditions the membrane may act either as a Cl or a K electrode (Hodgkin & Horowicz, 1959). Replacement of Cl by thiocyanate also increases the length of action potential and twitch in frog muscle (Lubin, 1957). Cl activates the enzyme amylase (cf. Bayliss, 1959, p. 308).



*Ion antagonism*

Some of the effects of individual ions on metabolic function have been considered already, but in many cases the actions of different ion species are mutually antagonistic. It is therefore important that correct ionic ratios should be present in bathing fluids. Certain actions of the cations are dependent on their valency, as for instance the dehydration of lipids by divalent ions such as Ca and Mg, an effect antagonized by the monovalent ions Na and K. In other reactions Na and K and Ca and Mg are antagonistic to each other.

Differences occurring in the effect of the Na : K ratio on the same organ from different animals appear to result from the relative sensitivities of the integral components of the organ. Thus the effect of high and low Na and K on the rate of beat, amplitude and tonus of various vertebrate and invertebrate hearts depends on the relative sensitivity of pacemaker, contraction mechanism and conducting tissue (Prosser *et al.*, 1950). Similarly the antagonistic action of Ca on hearts is related to its effect on these three systems. Factors other than valency are also involved, Mg is less effective than Ca in causing muscle contraction but has a similar action to that of Ca on the pacemaker.

Many types of nervous tissue are sensitive to the K : Ca balance. Low Ca or high K concentration initially stimulates many vertebrate and invertebrate ganglia. Conversely high Ca and low K decrease the discharge of autonomic ganglia. The K : Ca ratio is also important in synaptic transmission. Ca and Mg are mutually antagonistic at the neuromuscular junction where Mg decreases and Ca increases the output of ACh (Hutter & Kostial, 1954).

Ashford & Dixon (1935) find that the addition of extra K to a balanced saline increases the $O_2$ consumption of tissues. This is antagonized by Ca and Mg, both of which depress $O_2$ utilization (Dickens & Greville, 1935).

Further examples of interaction between Ca and K include inhibition of the movements of the foregut of *Dytiscus* by K, an effect antagonized by Ca (Hobson, 1928), and a mutually antagonistic effect on the spontaneous activity of the C.N.S. of the crayfish (Roeder, 1941).

Ion antagonism is discussed in more detail by many authors including Fruton & Simmonds (1958), Prosser *et al.* (1950) and Heilbrunn (1952).

*Hydrogen ion*

The pH of the blood of most animals is in the range 6·8–7·6, though in some insects and a number of forms from highly acidic waters it may be as low as 6·1. Some marine invertebrates on the other hand, particularly molluscs, have a pH of about 7·6–7·9.

The pH of the medium bathing tissues affects such factors as rate of ion transport, colloidal O.P. enzyme activity, inhibition of water by gels, etc.

The chemical properties of proteins change with the pH since proteins are ampholytes behaving as acids on the alkaline side of the isoelectric point and as alkalies on the acidic side. Furthermore the hydration of proteins is regulated by the pH, the water content decreasing to a minimum at the isoelectric point. The effect of the hydrogen ion on the hydration of proteins is however modified by the presence of other ions. Enzymic activity is usually at a maximum close to the isoelectric point and it appears that catalytic activity is associated with an absence of a net charge on certain specific groups (cf. Bayliss, 1959, p. 308).

The optimum pH for the uptake of K by K-depleted red blood corpuscles is optimal at pH 7·4 (Ponder, 1950) and the same applies to the extrusion of Na (Harris & Maizels, 1951).

Raising the pH of the fluid in which muscles are bathed results in K transfer to the cells and, conversely, if the solution is made more acid an exchange of H for Na and K occurs which tends to decrease the net alkali metal ion content of the cells (cf. Darrow & Hellerstein, 1958).

As the tissues of many animals are markedly affected by deviations of only 0·1–0·2 units of pH from the normal haemolymph value, salines should be buffered as closely as possible to the natural value.

*Osmotic pressure*

One of the best examples of the interaction of ions in a metabolic system is that involved in the maintenance of the water balance of cells. Slices of mammalian tissues, incubated under conditions which depress metabolism, take up fluid from salines and hypertonic solutions (Itoh & Schwartz, 1956); but when the metabolic activity is restored the swollen tissues lose fluid and return to approximately their normal volume. The maintenance of the water content of cells is thus to be regarded as a dynamic rather than a passive process. The current explanation of the maintenance of water balance is given by Robinson (1960) in terms of the ionic metabolism. Sodium diffusing down the electrochemical gradient into cells is actively pumped out and the cell membrane thus behaves as if it were effectively

impermeable to sodium. If the metabolism of the cell is impaired, Na is not excluded as fast as it enters and K tends to be lost in exchange for Na. In the normally functioning cell Cl entry down the concentration gradient is excluded by the membrane potential and when this potential falls, as a result of K loss from the cell, Cl enters. Water was previously prevented from entering the cell by the double Donnan effect of the exclusion of Na counteracting the colloidal osmotic pressure* of the organic constituents. On the entry of Na and Cl into the cell, water is also able to enter and the cell swells. Breakdown and death follow if normal metabolic exclusion of Na is not resumed. Thus, as Robinson points out, there is an apparently anomalous situation in which a saline is osmotic with cell contents and is only isotonic when the cell is metabolizing normally. Any saline designed to maintain the water and ionic contents of cells in a normal state should therefore incorporate a respiratory substrate, unless adequate food reserves are known to be present in the tissue.

The permeability of cells to water is greater than that to ions (cf. Dick, 1959) but the cell O.P., being in a dynamic steady state, is unlikely to be exactly the same as that of the extracellular fluid though it tends towards it. All other factors excluded, therefore, the cell water content would be expected to vary inversely with the O.P. of the extracellular fluid, a factor of some importance as the O.P. and Na of many physiological salines differ rather widely from the corresponding levels in blood. Fortunately some degree of dynamic control is exerted over the colloid O.P. of cells of most animals. In vertebrates a smaller increase in the water content than expected occurs in muscles exposed to hypotonic saline for 24 hr, whilst heart, liver and brain show little swelling in this time (cf. Darrow & Hellerstein, 1958). The fact that exposure of these tissues to the same hypotonic salines for periods of less than 24 hr results in a larger water uptake suggests that the contribution to the colloid O.P. of the cell may be varied. The dibasic-acid content of muscles has been found to increase in cases where Na + K in cells are at a sub-normal level as a result of induced alkalosis (cf. Darrow & Hellerstein, 1958). A similar but more extensive change in intracellular amino-acid content in response to changes in the O.P. of the extracellular fluid has been found in several brackish-water Crustaceans and Molluscs (Potts, 1958; Shaw, 1958) and may be inferred from changes in the Cl content of cells in other forms (Dresco-Derouet, 1959; Beadle & Cragg, 1940). Slight inaccuracies in maintaining the O.P. of physiological salines precisely the same as in cells is thus unlikely to have serious deleterious effects on metabolic function, though the composition of the components responsible for the cell O.P. may undergo some change. The level of O.P. of the bathing fluid has a specific effect on some tissues, however. Thus Hughes & Kerkut (1956) found that the spontaneous activity of *Arion* nerve cord varied with the O.P. of the bathing medium whilst excess NaCl in the bathing medium results in a fall in the resting potential, increased spike frequency and increased tension in the guinea-pig's gut (Holman, 1957).

* The term colloidal osmotic pressure is used here to indicate the osmotic pressure due to non-penetrating organic substances.

## 3. PHYSIOLOGICAL SALINES, BLOOD COMPOSITION AND CELL FUNCTION IN THE MAIN ANIMAL GROUPS

In the account that follows, details are given of some of the physiological salines in use with the isolated tissues of the major vertebrate and invertebrate groups. Where analyses of the blood ion concentrations are known for the invertebrate species, these are given for comparison with the composition of the salines. Mention of the properties of tissues in relation to the ionic environment is made where these differ from that already discussed. Naturally, however, the selection of such data from the considerable literature tends to be somewhat arbitrary.

### *Vertebrates*

The first satisfactory physiological saline for vertebrates was developed by Ringer (1883a) as a result of work on the effect of ions on the isolated frog's heart. He demonstrated the necessity for a balanced relationship between the concentrations of Na, K and Ca in the medium bathing this tissue. Locke (1901) modified Ringer's solution to render it suitable for mammals by raising the osmotic pressure and adding glucose as a respiratory substrate; Tyrode (1910) further added magnesium and phosphate for use with the mammalian gut. Tyrode's solution, with small modifications, has been found one of the most useful for bird and mammal tissues, though it suffers both from a liability to become too alkaline and from calcium precipitation.

As much of the work described in the section on the effects of ions on tissues has been elucidated on vertebrate tissues no further general comment is required. The O.P. of various species of Amphibia, reptiles, birds and mammals are listed below together with some of the more satisfactory salines. Fish are considered separately.

### *Amphibia*

Amphibian tissues have received considerable attention at the hands of physiologists and many variations of the original Ringer solution have been developed to potentiate special features of different tissues. Adequate buffering is present in most of the salines used for general purposes. One of the best of these, prepared by Boyle & Conway (1941), is based on blood analyses which make allowance for the partial binding of Ca and Mg onto organic components of haemolymph. Frog nerve and muscle preparations tend to lose K when isolated and the K and Ca contents of salines should perhaps be raised somewhat to minimize this. Harris (1953) describes a bicarbonate-rich saline which maintains the K content of frog muscle. *Bufo* nerves appear to be more resistant to K loss and Shanes & Burman (1955) did not observe any loss of K from their preparations over a period of 48 hr despite the fact that their saline has a rather low $CaCl_2$ level and no bicarbonate. A high Ca and K level is required for the maintenance of the melanophores of frog skin (Horowitz, 1958) but the K concentration must be low for the maintenance

of the resting potential of muscle (Ling & Gerard, 1949). Variations in the composition of salines for special purposes include that by Conway *et al.* (1959) for loading muscles with K and for allowing their subsequent recovery, and by Whitcomb *et al.* (1958) for modifying the pH.

Unbuffered salines and those which are buffered by bicarbonate only are liable to pH change and should not be used in long-term experiments.

One gram per litre glucose may be added to the solutions listed in Table 1 where not otherwise specified.

Table 1—The composition of

Salt concentrations are given in terms of grams/litre. Values

Frog blood (mM/kg plasma)

| Authority | Na | K | Ca | Mg |
|---|---|---|---|---|
| Fenn (1936) | 103·8 | 2·5 | 2·0 | 3·0 |



Salines

| Authority | Tissue | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | $NaHCO_3$ | $Na_2HPO_4$ |
|---|---|---|---|---|---|---|---|---|
| Ringer (1883b) | Heart | 6·5 | 0·14 | 0·12 | — | — | 0·2 | — |
| Boyle & Conway (1941) | Muscle | 4·24 | 0·148 | — | — | 0·146 | 2·1 | 0·356 |
| Forster (1948) | Kidney | 5·8 | 0·19 | 0·22 | 0·2 | — | 1·26 | — |
| Harris (1953) | Muscle | 5·26 | 0·22 | 0·22 | — | 0·12 | 2·52 | 0·33 |
| Horowitz (1958) | Melanophores | 6·5 | 0·25 | 0·30 | — | — | — | — |
| Ling & Gerard (1949) | Muscle | 6·5 | 0·12 | 0·14 | — | — | 0·2 | — |

*Mammals and birds*

The solutions listed in Table 2 have been developed as perfusates for birds' and mammals' tissues or as diluents of sera. When preparing the salines each salt should be added in the order given in the table and each should be fully dissolved before the next is added. Tyrode and other solutions containing bicarbonate show a tendency to lose $CO_2$ and become more alkaline. They should not be sterilized by boiling or precipitation of calcium is likely. Sterilization of such solutions should be by filtration or, if by boiling, prior to the addition of bicarbonate. As an alternative to Tyrode, Fleisch's solution may be used, the two components of which can be boiled separately and then mixed. It is not advisable to store Tyrode and other bicarbonate salines, but if they must be kept temporarily they should be contained in glass or rubber stoppered bottles in order to prevent the loss of $CO_2$. Salines containing $PO_4$ and glucose must be stored in a refrigerator

to minimize bacterial action, though it is preferable that these substances should only be added to the saline immediately prior to use.

In the event of Tyrode solution becoming too alkaline the pH may be readjusted by the addition of dilute hydrochloric acid.

For further information and technical hints on the preparation of vertebrate salines see Parker (1950), Cameron (1935) and Strangeways (1924). Salines for use with the Warburg technique are discussed by Cohen in Umbreit (1949) and Krebs (1950). Krebs also lists the components of human blood plasma and

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are calculated as amorphous salts unless otherwise stated.

Frog blood (mM/kg plasma)—*continued*

| Cl | $HCO_3$ | Total phosphate | pH | Δ°C |
|---|---|---|---|---|
| 74·3 | 25·4 | 3·1 | 7·4–7·8* | 0·395 |

Salines—*continued*

| $NaH_2PO_4$ | $KH_2PO_4$ | $Na_2SO_4$ | Ca gluconate | Glucose | Notes |
|---|---|---|---|---|---|
| — | — | — | — | — | — |
| — | 0·068 | 0·092 | 0·4 | 4·684 | Equilibrate with 97% $O_2$ and 3% $CO_2$. Cellular swelling is less than in Barkan's saline. |
| 0·07 | — | — | — | 0·54 | — |
| — | — | — | — | — | For maintenance of muscle K at 18°C. Bubble 95% $O_2$+5% $CO_2$. |
| 0·02 $NaH_2PO_4$ and $Na_2HPO_4$+0·1 N NaOH to pH 7·4 | | | | | — Melanophores survive for 48 hr if solution is changed every 12 hr. |
| — | — | — | — | — | For maintenance of muscle-resting potential. |

* Ling & Gerard (1949).

suggests some organic additions which should be made to the inorganic composition of vertebrate salines.

*Reptiles*

No salines seem to have been designed specifically for use with reptilian tissues. The few analyses available for the ionic composition of the haemolymph suggest, however, that mammalian salines should be adequate, if the total concentration is appropriately adjusted.

Analyses of the blood of snakes are given by Hutton (1958) (*Coluber*, *Lampropeltis*, *Natrix* and *Agkistrodon*); Carmichael & Petcher (1945) (Rattlesnake); Luck & Keeler (1929) (Rattlesnake). Turtle blood has been analysed by Hutton & Goodnight (1957) and by Smith (1929). Hernandez & Coulson (1951) have

TABLE 2(a)—MAMMALIAN AND

The composition of salines is given in grams/litre. Values are calculated for

| Authority | | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | $NaHCO_3$ | $Na_2HPO_4$ |
|---|---|---|---|---|---|---|---|---|
| Tyrode (1910) | | 8·0 | 0·2 | 0·2 | 0·05 | — | 1·0 | — |
| Earle (1943) | | 6·8 | 0·4 | 0·2 | — | 0·1 | 2·2 | — |
| Fleisch (in Gatenby, 1937) | A | 8·0 | 0·2 | — | — | — | 20 ml N | — |
| | B | — | — | 0·2 | 0·1 | — | — | — |
| Hanks (in Umbreit, 1949) | (1) | 8·0 | 0·4 | 0·2 | 0·035 | 0·05 | 0·34 | 0·06 |
| Hanks (1948) | (2) | 8·0 | 0·4 | 0·2 | — | 0·1 | — | 0·08 |
| Krebs & Henseleit (1932) | (1) | 7·44 | 0·38 | 0·3 | — | 0·16 | — | 1·4 |
| Krebs & Henseleit (1932) | (2) | 6·92 | 0·35 | 0·28 | — | 0·15 | 2·1 | — |
| Lewis (1911) | | 7·0 | 0·42 | 0·25 | — | — | 0·2 | — |
| Locke (1901) | | 9·0 | 0·42 | 0·24 | — | — | 0·2 | — |
| Pannet & Compton (1924) | | | | | | | | |
| | A | 12·11 | 1·55 | 0·77 | 0·63 | — | — | — |
| | B | — | — | — | — | — | — | 55 ml $\frac{M‡}{69}$ |
| Sims & Saunders (1942) | | 8·0 | 0·2 | 0·147* | 0·203† | — | 1·01 | 0·213 |
| White (1949) | | 7·0 | 0·375 | — | — | 0·275 | 0·55 | 0·058 |
| Drew (in Strangeways, 1924) | (1) | 9·0 | 0·42 | 0·2 | — | 0·1 | 0·2 | $CaH_4(PO_4)_2$ + 0·1 |
| Drew (in Strangeways, 1924) | (2) | 9·0 | 0·42 | 0·2 | — | — | 0·2 | $CaH_4(PO_4)_2$ 0·1 |
| Gey (1949) | | 8·0 | 0·38 | 0·13 | 0·1 | — | 0·25 | 0·12 |

* $CaCl_2.2H_2O$. † $MgCl_2.6H_2O$. ‡ $Na_2HPO_4.12H_2O$.

TABLE 2(b)

Krebs (1950) has developed three solutions for respiratory studies bicarbonate, high phosphate and low phosphate and bicarbonate precipitation of calcium phosphate (Table 2b).

| | 0·9% NaCl (parts) | 1·15% KCl (parts) | 0·11 M $CaCl_2$ (parts) | 2·11% $KH_2PO_4$ (parts) |
|---|---|---|---|---|
| High bicarbonate | 80 | 4 | 3 | 1 |
| High phosphate | 83 | 4 | – | 1 |
| Low bicarbonate and low phosphate | 95 | 4 | 3 | 1 |

To each of these three solutions add 7 parts 0·1 M Na-fumarate, 4 parts 0·16 M

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amorphous salts unless otherwise stated

| $NaH_2PO_4$ | $KH_2PO_4$ | Glucose | Water | pH | Notes |
|---|---|---|---|---|---|
| 0·04 | — | 1·0 | 1000 | 6·8–7·2 | Must not be sterilized by boiling. Loss of $CO_2$ results in pH change on standing. |
| 0·11 | — | 1·0 | 1000 | — | High bicarbonate present, sterilize by filtration not boiling. |
| — | — | — | to 500 | — | To sterilize boil solutions A and B separately, cool and mix. |
| 3·5 ml N $H_3PO_4$ | — | 1·0 | (8 ml N HCl) to 500 | | |
| — | 0·06 | 1·0 | 1000 | — | Diluent for sera. |
| — | 0·1 | 2·0 | to 1000 | — | For bird heart. After sterilizing by boiling add 100 ml 1·4% $NaHCO_3$ to each litre. |
| — | 0·17 | — | 1000 | — | For respiratory studies on homogenates. |
| — | 0·16 | 2·0 | 1000 | — | Gas with 5% $CO_2$ and store in glass topped bottles. |
| — | — | 2·5 | 900 | — | For poikilothermic vertebrates. |
| — | — | 1·0 | to 1000 | — | |
| — | — | — | | | Add 4 ml A to 6 ml B and make up to 100 ml with distilled water. This saline is too dilute for chicken tissues (Howard, 1953). |
| 5 ml $\frac{M}{69}$ | — | — | | | |
| — | — | — | to 1000 | — | Serum diluent. |
| — | 0·026 | 8·5 | 1000 | — | Plus 0·21 g $Ca(NO_3)_2 2H_2O$ and 0·001 g $Fe(NO_3)_3$. This saline is more effective than Tyrode or Locke saline for bird tissue. |
| — | $MgHPO_4$ 0·1 | — | 1000 | — | |
| — | $MgHPO_4$ 0·1 | — | 1000 | — | |
| — | 0·025 | 1·0 | 1000 | — | |

KREBS SOLUTIONS

on tissue slices and homogenates. These have respectively a high content. Calcium is omitted from the high phosphate saline to avoid

| 3·82% $MgSO_4 7H_2O$ (parts) | 1·3% $NaHCO_3$ (parts) | 1·78% $Na_2HPO_4$ (parts) | 0·1 M $NaH_2PO_4$ (parts) |
|---|---|---|---|
| 1 | 21 | — | — |
| 1 | 3 | 14·4 | 3·6 |
| 1 | 3 | 2·4 | 0·6 |

Na-pyruvate, 4 parts 0·16 M Na-glutamate and 5 parts 0·3 M glucose (5·4 per cent).

worked on Iguana blood composition. Some representative freezing point depressions are listed in Table 3.

TABLE 2C—THE OSMOTIC PRESSURE OF SOME REPRESENTATIVE MAMMALS AND BIRDS, Δ°C

| Animal | Δ°C | Authority |
|---|---|---|
| Sheep | 0·59 | Aldred (1940) |
| Dog | 0·56 | Aldred (1940) |
| Goat | 0·57 | Aldred (1940) |
| Horse | 0·56 | Aldred (1940) |
| Cow | 0·56 | Aldred (1940) |
| Man | 0·57 | Aldred (1940) |
| Seal | 0·545–0·614 | Page *et al.* (1954) |
| Chicken | 0·55 | Howard (1953) |
| Pigeon | 0·61 | Aldred (1940) |
| Hen | 0·61 | Aldred (1940) |
| Duck | 0·55–0·57 | Aitkins (1909) in Howard (1957) |
| Goose | 0·55–0·57 | Aitkins (1909) in Howard (1957) |

TABLE 3

| Species | Δ°C | Authority |
|---|---|---|
| *Emys europa* | 0·44 | Bottazzi (1908) |
| *Testudo graeca* | 0·60 | Burian (1910) |
| *Caretta kempi* | 0·66 | Smith (1932) |
| *Caretta caretta* | 0·76 | Smith (1932) |
| "Tortoise" | 0·57 | Aldred (1940) |

*Fish*

The assumption is frequently made that the osmotic pressure of F-W teleosts is equivalent to about 0·16 M NaCl and marine teleosts to about 0·22 M NaCl. Salines with these approximate concentrations have been used indiscriminately without taking into account the actual O.P. of experimental fish. In fact a considerable range of concentration occurs and Green & Hoffman (1953) determining the concentration of NaCl isotonic with the haemocytes of various teleosts obtained values from 0·1 to 0·26 M. It should be remembered, however, that it has repeatedly been shown that, in the absence of calcium, red cells of many groups of animals tend to lose potassium and gain NaCl, thus disturbing the osmotic balance (cf. Maizels, 1954). The isotonic levels found by Green & Hoffman are thus to be regarded as distinct from those in balanced saline and as a guide only to the variation in the osmotic pressure of the fish studied. Measured depressions of freezing points for fresh-water teleosts range from 0·45°C *Tinca* (Dekhuysen, 1905) to 0·64°C *Salmo* (Benditt *et al.*, 1941) and in marine teleosts from ≡ 0·60°C *Gasterosteus* (Koch & Heuts, 1943) to 1·13°C *Mackerel* (Green & Hoffman, 1953). Measured Δ's for a range of fish are reproduced in Table 5 and further lists will be found in Brown (1957), Heilbrunn (1952) and Smith (1932).

The recorded pH of fish blood lies in the range 6–7·8, but it has been suggested by Robertson (1954) that the lower values which were found by Willmer (1934) may be in error. For pH of individual species see Brown (1957).

Robertson (1954) has made a careful analysis of the ion concentrations in the blood of a fresh-water and marine teleost (Table 6) which indicates that, whilst all the ions except K are lower in the fresh-water *Coregonus* than the marine *Muraena*, the greatest fall is in NaCl. Mere alteration of the water content of a saline to adjust the concentration to isotonicity with the blood of fish is therefore inadvisable and, in the absence of a complete analysis of the ions, it would be more desirable to adjust only the NaCl content. Probably the salines most likely to be successful, with the NaCl content suitably adjusted, are those of Keynes & Martins-Ferreira (1953) for F-W teleosts and Young (1933) for marine teleosts, though some reduction of the Mg content might be advantageous. Young's saline for elasmobranchs buffered with $NaHCO_3$ and $KH_2PO_4$ as in the quantities given for *Lophius* appears satisfactory for this group.

*Vertebrate embryos and sperm*

The body fluids of all vertebrate embryos studied are more dilute than those of the corresponding adult (Adolf, 1957; Howard, 1957) and in some cases the relative ion concentrations also change with age [Forbes (1955) quoted by Adolf (1957)]. In the case of the chick, Howard finds a gradual increase in the O.P. from $\Delta$0·425°C to $\Delta$0·57°C of the egg white, sub-germinal fluid, blood and amniotic fluid from the time of laying till hatching of the egg. A similar ontogenetic rise in O.P. occurs in the trout and goose, whilst even in the mammal embryo Na + K is some 14 per cent below the maternal level (Adolf, 1957). The NaCl level of salines for use with embryonic tissues should thus be diluted appropriately from the level suitable for adult tissues.

The best known salines for embryos are those used for Amphibia. Holtfreter is the better known of these but the solution developed by Nieu has the advantage that it can be sterilized and also that the pH is less alkaline than that of the former solution (Flickinger, 1949).

Diluents for mammalian sperm are discussed by Rothschild (1959), who finds that Baker's glucose saline and his own saline R are the most suitable. Ryle (1957) finds that Baker's glucose saline is also adequate for avian sperm.

The $\Delta$ for Holtfreter of 0·67°C given by Richards (1940) is clearly erroneous but that of 0·25°C found by Tuft (personal communication) is close to the value expected from the chemical composition.

*Artificial sea-water*

Tables giving the concentrations of ions in sea-water of different salinities are given by Barnes (1954), and the relative vapour pressures of the ions by Robinson (1954). The solutions based on these and other analyses are given in Table 8.

Dilutions of sea-water should be made with M/400 $NaHCO_3$ as this ensures the maintenance of a constant pH at about the sea-water value (Wells & Ledingham,

TABLE 4—
Values are given in grams/litre, and

| Authority | Animal | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $NaHCO_3$ | $Na_2HPO_4$ |
|---|---|---|---|---|---|---|---|
| Cyclostomata | | | | | | | |
| Fänge (1948) | *Myxine* | 22·0 | 0·4 | 0·5 | 0·4 | 0·3 | — |
| Young (1933) | *Petromyzon* | 5·5 | 0·14 | 0·12 | — | — | — |
| Elasmobranchs | | | | | | | |
| Bialaszewicz (1933) | *Scyllium* | 15·8 | 0·84 | 0·52 | 0·65 | — | — |
| Feldberg & Fessard (1942) | *Torpedo* | 20·0 | 1·0 | 0·83 | 0·83 | 0·17 | — |
| Lutz (1930) | "Elasmobranchs" | 16·38 | 0·596 | 0·555 | — | 0·168 | — |
| Nichols in Gatenby (1937) | "Elasmobranchs" | 16·38 | 0·89 | 1·11 | — | 0·38 | — |
| Young (1933) | "Selachians" | 22·0 | 0·52 | 0·44 | 0·47 | — | — |
| Teleosts (marine) | | | | | | | |
| Forster & Hong (1958) | Flounder | 7·8 | 0·18 | 0·166 | 0·095 | 0·084 | — |
| Wasserman *et al.* (1953) | Flounder | 7·84 | 0·19 | 0·17 | 0·095 | 3·36 | — |
| Young (1933) | *Lophius* | 12·0 | 0·6 | 0·25 | 0·35 | 0·19 | — |
| Young (1933) | *Uranoscopus* | 13·5 | 0·6 | 0·25 | 0·35 | — | — |
| Teleosts (fresh-water) | | | | | | | |
| Altamiro & Coates (1957) | *Electrophorus* | 11·1 | 0·37 | 0·33 | 0·14 | — | — |
| Holmes & Stott (1960) | *Salmo clarkii* | 7·41 | 0·36 | 0·17 | — | 0·31 | 1·6 |
| Keynes & Martins-Ferreira (1953) | *Electrophorus* | 9·88 | 0·37 | 0·33 | 0·14 | — | 0·17 |
| Schoffeniels (1960) | *Electrophorus* | 9·36 | 0·37 | 0·67 | 0·14 | — | — |
| Young (1933) | "Freshwater teleosts" | 6·5 | 0·14 | 0·12 | — | — | — |

FISH SALINES
are calculated as anhydrous salts

| $NaH_2PO_4$ | $KH_2PO_4$ | $MgSO_4$ | Urea | Water | Notes |
|---|---|---|---|---|---|
| Cyclostomata—*continued* | | | | | |
| — | — | — | — | to 1000 | Probably hypotonic to *Myxine* blood but if refrigerated gut activity is retained for 4 days, pH 7·8. |
| — | — | — | — | to 1000 | Buffer with $NaHCO_3$ or phosphate. |
| Elasmobranchs—*continued* | | | | | |
| — | — | $Na_2SO_4$ 0·56 | 23·6 | to 1000 | |
| — | — | — | 25·0 | to 1100 | Replacement of Urea by NaCl results in pronounced oedema. |
| — | — | — | 21·6 | 1000 | |
| 0·06 | — | — | 21·6 | 1000 | |
| — | — | — | 29·0 | to 1000 | Buffer with $NaHCO_3$ or phosphate. |
| Teleosts (marine)—*continued* | | | | | |
| 0·06 | — | — | — | to 1000 | Phosphate buffered solutions without bicarbonate do not give maximal renal tubule transport. |
| 0·06 | — | — | — | to 1000 | |
| — | 0·068 | — | — | to 1000 | |
| — | — | — | — | to 1000 | Buffer with phosphate or bicarbonate. |
| Teleosts (fresh-water)—*continued* | | | | | |
| — | — | — | — | to 1000 | Probably too hypertonic. For effect of varying ionic composition see Altamiro & Coates (1957). |
| 0·4 | — | 0·15 | — | to 1000 | Isosmotic with blood Δ 0·58°C, pH 7·2. |
| 0·04 | — | — | — | to 1000 | Based on blood analyses by Hargeaves & Moreia (1949). |
| — | — | — | — | to 1000 | |
| — | — | — | — | to 1000 | Buffer with phosphate or bicarbonate. |

See also the *Manual of Formulae and Methods III* of the Marine Biological Laboratory, Woods Hole.

1940). Where it is desired to change the ionic balance of artificial sea-water various authors have found it an advantage to make up stock solutions of the individual constituent salts isotonic with sea-water. The ionic concentrations may then be varied as desired by mixing, without appreciably altering the osmotic pressure of the medium (Pantin, 1934; Wells & Ledingham, 1940).

TABLE 5—THE O.P. OF THE HAEMOLYMPH OF SOME REPRESENTATIVE FISH

| Species | Medium | Haemolymph O.P. (Δ°C) | Authority |
|---|---|---|---|
| *Cyclostomata* | | | |
| *Petromyson marinus* | — | 0·55 | Hardisty (1956) |
| *Lampetra fluviatilis* | Fresh-water | 0·456 | Morris (1958) |
| *Lampetra planeri* | Fresh-water | 0·40 | Hardisty (1956) |
| *Holostii* | | | |
| *Amia calva* | — | 0·54 | Smith (1932) |
| *Lepidosteus* | — | 0·57 | Smith (1932) |
| *Teleostii* | | | |
| *Anguilla rostata* | Fresh-water | 0·63 | Smith (1932) |
| *Anguilla rostata* | Sea-water | 0·82 | Smith (1932) |
| *Salmo salar* | Fresh-water | 0·64 | Benditt *et al.* (1941) |
| *Salmo clarkii* | Fresh-water | 0·58 | Holmes & Stott (1960) |
| *Gasterosteus* | Fresh-water | *ca* 0·60 | Koch & Heuts (1943) |
| *Gadus virens* | Sea-water | 0·76 | Dekhuysen (1905) |
| *Lophius piscatorius* | Sea-water | 0·67 | Forster (1953) |
| *Conger vulgaris* | Sea-water | 0·77 | Duval (1925) |
| *Myoxocephalus octodecimspinosus* | Sea-water | 0·715 | Forster (1953) |
| *Scomber scomber* | Sea-water | 1·13 | Green & Hoffman (1953) |
| *Tautoga onitis* | Sea-water | 1·05 | Green & Hoffman (1953) |

TABLE 6—A COMPARISON OF THE IONIC COMPOSITION OF THE HAEMOLYMPH OF FOUR FISH

Values as mM/Kg

| Genus | Na | K | Ca | Mg | Cl | $SO_4$ | $HCO_3$ | Authority |
|---|---|---|---|---|---|---|---|---|
| *Lampetra* | 119·6 | 3·21 | 3·93 | 4·21 | 95·9 | 5·44 | 6·41 | Robertson (1954) |
| *Muraena* | 211·8 | 1·95 | 7·73 | 4·85 | 188·4 | 11·35 | 8·03 | Robertson (1954) |
| *Coregonus* | 140·9 | 3·81 | 5·34 | 3·38 | 116·8 | 4·58 | 10·58 | Robertson (1954) |
| *Lophius* | 228 | 6·4 | 2·3 | 3·7 | 164 | — | — | Edwards & Condorelli (1926) |

Further data on the O.P. and ionic composition of the blood of various fish will be found in Brown (1957) and Prosser *et al.* (1950).

Trace elements in sea-water are given by Webb (1937), and Hale (1957) discusses the requirements of organisms in marine aquaria. Further data on the culturing of marine organisms are given by Galtsoff *et al.* (1937), and they also list the older formulae for sea-water by McClendon, Penn, Von Flach and Schwatz.

Nitrates and phosphates must be added if marine algae are to be cultured and the latter should be an advantage where artificial sea-waters are used as physiological salines.

TABLE 7—SALINES FOR AMPHIBIAN EMBRYOS
Values as grams/litre

| | NaCl | KCl | $CaCl_2$ | $MgSO_4$ | $NaHCO_3$ | $Na_2HPO_4$ | $KH_2PO_4$ | Water | pH |
|---|---|---|---|---|---|---|---|---|---|
| Holtfreter (1943) | 3·5 | 0·05 | 0·1 | — | — | — | — | 1000 | 8·2 |
| Nieu in Flickinger (1949) A* | 3·4 | 0·05 | $Ca(NO_3)_2 4H_2O$ 0·08 | 0·1 | — | — | — | 500 | 7·8 |
| B | — | — | — | — | 0·2 | — | — | 250 | |
| C | — | — | — | — | — | 0·11 | 0·02 | 250 | |

* Sterilize A, B and C separately and mix after cooling.

*Annelids*

Sea-water is a satisfactory medium for the isolated tissues of most marine worms. Wells & Ledingham (1940) have shown that the gut, body wall, nerve cord and nephridial cilia of *Arenicola* can be kept alive for many hours in this medium and that these tissues can also tolerate wide changes in the osmotic concentration of the medium. *Arenicola* heart also survives well in sea-water (Prosser & Zimmerman, 1943) but Prosser & Melton (1954) find that *Phascolosoma* proboscis muscle survives better in Hodgkin & Katz artificial sea-water. Shock inhibition of activity occurs when *Arenicola* and *Nereis* tissues are suddenly transferred to hypotonic solutions but activity is regained some hours later if the medium is within the physiological range of tolerance (Wells & Ledingham, 1940). Recovery of activity is apparently related to regulation of tissue ionic content rather than water content, as the tissues remain swollen in hypotonic media.

Terrestrial worms are also tolerant of considerable changes in the osmotic concentration of their body fluids, *Lumbricus* recovering after up to 70 per cent of the total body water has been lost by evaporation (Roots, 1956). This osmotic tolerance presumably accounts for the finding of Collier (1939) that diluting frog ringer to isotonicity with earthworm blood did not result in any additional survival of nerve, and also for the reasonable success of the mammalian saline used by Rushton (1945) in which the nerve cord survived for 24 hr. Frog ringer has also been used for earthworm gut (Millott, 1942), heart (Ambache *et al.*, 1945) and nerves (Taylor, 1940). The heart survives for up to 12 hr in frog ringer. Analyses of the blood and coelomic fluid by Bahl (1947) and by Maluf (1940) indicate, however, that earthworms have considerably higher K/Na and Ca/Na

TABLE 8—COMPOSITION OF SOME ARTIFICIAL SEA-WATERS
Salts in grams/litre

| Authority | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $Na_2SO_4$ | NaBr | $NaHCO_3$ | $SrCl_2$ | $H_3BO_3$ | NaF | KBr | pH | Salinity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hale (1957) | 23·991 | 0·72 | 1·135 | 5·102 | 4·012 | 0·085 | 0·197 | 0·011 | 0.027 | — | — | — | 34·33 |
| Lyman & Flemming (1940) | 23·477 | 0·664 | 1·102 | 4·981 | 3·917 | — | 0·192 | 0·024 | 0·026 | 0·003 | 0·096 | 7·9–8·3 | 34·38 |
| Vant Hoff | 25·7 | 0·67 | 1·0 | 1·9 | — | — | to pH 8·2 | — | — | — | — | 8.2 | |
| Hodgkin & Katz (1949) | 25·3 | 0·73 | 1·14 | 4·85 | — | — | 0·21 | — | — | — | — | — | — |
| Scheer & Scheer (1951) | 22·9 | 0·7 | 1·1 | 4·7 | 3·8 | 0·082 | — | — | 0·011 | — | — | KOH to pH 8·2 | — |
| Pantin (1934) | 31 | 0·99 | 1·37 | 2·35 | — | — | 0·22 | — | — | — | — | to pH 7 | — |
| Van Harreveld & Russell (1954) | 25·7 | 0·67 | 1·0 | 1·9 | — | — | — | — | — | — | — | — | — |
| Wells & Ledingham (1940) | 26·46 | 0·8 | 1·24 | 5·5 | 4·29 | — | — | — | — | — | — | — | — |
| Pantin (1946) | 23·427 | 0·729 | 1·124 | 5·013 | 3·953 | 0·059 | 0·210 | — | — | — | — | — | — |
| Robertson & Webb (1939) | 23·4651 | 0·7246 | 2·2040* | 10·6364† | 3·9282 | — | — | — | — | — | — | — | — |

* As $6H_2O$. † As $6H_2O$.

ratios than the vertebrates and the modification of the salines accordingly should improve them. In addition magnesium should be included as Wells & Ledingham have found that the activity of the isolated extrovert of *Arenicola* is related to the concentration of this ion.

Of the salines listed those of Pantin (1946) and Prosser & Zimmerman (1943) ought to be the most successful.

*Crustacea*

Sea-water is frequently used as a saline for the tissues of marine crustacea. Most of the more active crustaceans whose blood has been analysed have more K and less Mg and $SO_4$ in the blood than sea-water, however, and Cole (1941) has shown that the heart is sensitive to the Mg level and that sea-water is an unphysiological saline for this organ. A Mg concentration above the level in the blood decreases the rate and amplitude of heart beat in *Homarus* though conversely the absence of Mg from the bathing fluid also has an adverse effect on the heart (Cole, 1941). Bain (1929), however, finds that sea-water in which the pH has been lowered to 7·5 with HCl is adequate for use with the hearts of *Carcinus*, *Maia* and *Cancer* for experiments lasting several hours. In general the effect of ions on isolated tissues is the same as in other groups of animals, but *Carcinus* muscle is remarkable in that choline and some other quaternary ammonium ions can substitute for Na in the production of the muscle action potential (though the muscle cannot extrude choline and soon dies) (Fatt & Katz, 1953). Many tissues when isolated tend to lose K and take up NaCl and water, and Wright (1958) suggests that a similar movement of ions may be responsible for the loss of excitability of crustacean nerves kept at low temperatures. In view of the known palliative effect of raising the K and Ca concentrations of the medium on the ability of isolated tissues to maintain their K concentration, it is of interest that the best of the fifty-three media, tested as perfusates for the lobster heart, differed from the measured sera values principally in having raised K, Ca and Mg levels (Cole, 1941). As in other animals *Carcinus* nerves die rapidly in the absence of Ca (Hodgkin & Katz, 1949), but conversely the high Ca level in van Harreveld's solution does not adversely affect crayfish nerve (Solway *et al.*, 1953). Tissues of the crayfish, *Cambarus*, are intolerant of ionic concentrations differing markedly from those in the blood though withstanding pH variation in the range 6–8·4. Variations of only 5 per cent in the Ca level are reported to affect the heart beat and it is apparently for this reason that Lienemann's saline is much less satisfactory than that of van Harreveld which is based on the blood composition.

Cole's saline is unsatisfactory for use with lobster nerve as it renders the preparation hyperexcitable and less accommodating within about an hour (Wright & Rueben, 1958).

The effects of the ion ratios of the bathing medium on neuromuscular transmission are considered by Waterman (1941) and on the crustacean heart by Hogben (1925), Hogben & Hobson (1924) and Wells (1928). Bain (1929) discusses the effects of adrenalin and other drugs on the heart.

As in the case of other invertebrate groups, it is likely that the tolerance of the cells of Crustacea to changes in the O.P. of the bathing medium is related to the normal degrees of fluctuation occurring in the haemolymph of a species, as a result of changes in the concentration of the normal environment. Isolated tissues

Table 9—Composition of

Body fluid composition

Body fluid composition

| Authority | Animal | Na | K |
|---|---|---|---|
| Bahl (1947) | *Pheretima* | 41 | 19 |
| Maluf (1940) | *Lumbricus* | Na+K 89 | |
| Ramsay (1949) | *Lumbricus* | — | — |
| Rouchal (1940) | *Allobophora* | — | — |

Annelid salines

| Authority | Animal | Tissue | NaCl | KCl |
|---|---|---|---|---|
| Ambache *et al.* (1945) | *Lumbricus* | Heart | 6·8 | 0·14 |
| Pantin (1946) | Fresh-water leech | — | 6·6 | 0·32 |
| Pantin (1946) | Earthworm | — | 7·9 | 0·2 |
| Prosser & Zimmerman (1943) | Earthworm | Heart | 6·0 | 0·12 |
| Rushton (1945) | *Lumbricus* | Nerve cord | 8·0 | 0·2 |
| Roots (1955) | *Lumbricus* *Allobophora* | Cilia | 4·1 | 0·082 |

from the so-called conformers may be expected to adjust more readily and rapidly to media which are not isotonic than those from animals which normally regulate the haemolymph concentration within narrow limits. Furthermore, as many of the more efficient regulators maintain their haemolymph concentration hypotonic to sea-water when living in the sea, this medium should not be used with their tissues. Such forms include the grapsoid crabs and some prawns. The haemolymph concentration of a number of species is given in Table 11. The salines of Cole (1941), Pantin (1934) and Hagiwara & Bullock (1957) should prove suitable for these species if the NaCl concentration is decreased to render the saline isotonic with the normal haemolymph.

Numanoi (1937) and Robertson (1941) report that some of the calcium and magnesium in the blood is not dialysable and it is presumably bound to blood proteins. The magnesium concentration of salines based on the blood should thus be appropriately adjusted.

Some earlier salines prepared by Hogben (1925) (*Homarus*), Lienemann (1938) (*Cambarus*) and Zoond & Slome (1928) (*Palinurus*) are discussed by Cole *et al.* (1939) and Cole (1941).

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No specific salines have been prepared for other crustacea but such measurements as have been made of the composition of the blood of other forms indicate that the fresh-water forms have essentially similar ionic ratios in the blood though having a rather wide range of total concentration. Probably van Harreveld's or

ANNELID BODY FLUID AND SALINES

mM/l. Salines in grams/litre

Body fluid composition—*continued*

| Ca | Mg | Cl | $SO_4$ | Δ°C |
|---|---|---|---|---|
| 4·2 | 3·0 | 14 | — | 0·4–0·5 |
| 9·0 | — | 46 | 2·7 | 0·552 |
| — | — | 44 | — | 0·32–0·36 |
| — | — | — | — | 0·74 |

Annelid salines—*continued*

| $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | $NaHCO_3$ | $NaH_2PO_4$ | pH | Notes |
|---|---|---|---|---|---|---|
| 0·12 | — | — | 0·2 | 0·01 | 7·4 | |
| 0·2 | — | — | 0·14 | — | 7·4 | Plus phosphate buffer to pH 7·4 |
| 0·2 | 0·034 | — | 0·065 | — | 7·4 | |
| 0·2 | — | — | 0·1 | — | 7·4 | |
| 0·2 | — | 0·05 | — | — | — | |
| 0·084 | — | — | 0·28 | — | — | Cilia beat for up to 20 hr |

Prosser's salines would prove satisfactory for most fresh-water forms if the total concentration is adjusted to isotonicity with the blood of the experimental species. The ion concentrations of terrestrial forms are not well known, but in the vegetarian *Julus* (Diplopoda) the Na : K ratio is lower than in the fresh-water genera (Lockwood, unpublished). A short list of the depression of freezing point of the blood of some other lower arthropods is given as a guide to the preparation of isotonic salines (Table 11). For the ionic constituents of some of these forms and other crustacea see Prosser *et al.* (1950), Maluf (1939), Krogh (1939) and Waterman (1960).

*Molluscs*

Most of the salines in use with molluscan preparations have been developed for work on the isolated heart. In general the ionic composition has been arrived at empirically and Krijgsman & Divaris (1955) comment that the ionic ratios are more important than the total osmotic concentration—a point well illustrated by the diversity of concentrations in use. Osmotic tolerance for nerve has been found by Hughes & Kerkut (1956) (though the degree of spontaneous activity varies with

the O.P.) and the heart also shows some tolerance of changes in the total concentration (Barnard & Bonnet, 1930).

For marine species sea-water is commonly used but it has been found beneficial to adjust the pH nearer neutrality (Navez, 1936). The optimum pH for *Mytilus*

TABLE 10—HAEMOLYMPH COMPOSITION

Ions as mM/l and salts as g/l. Values

Haemolymph composition

| Authority | Animal | Na | K | Ca | Mg | Cl |
|---|---|---|---|---|---|---|
| Cole (1941) | *Homarus* | 454 | 9·3 | 18·6 | 9·0 | 472 |
| — | *Carcinus* | 515[2] | 12·3[2] | 12·9[2] | 17·0[1] | 490–545[3] |
| Robertson (1939) | *Cancer* | 502 | 12·0 | 13·8 | 27·4 | 509 |
| Schlatter (1941) | *Palinurus* | 531 | 12·0 | 19·5 | 10·4 | — |
| Buerger (1931) | | | | | | |
| Scholles (1933) | *Astacus* | 173–220 | 4·3–7·8 | 7·7–17·1 | 1·5–2·6 | 175–218 |
| Bryan (1960) | | (195) | (5·0) | (13) | (2·2) | (202) |
| Schlatter (1941) | *Cambarus* | 160 | 5·8 | 12·25 | 2·0 | — |

Salines

| Authority | Animal | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | $NaHCO_3$ | NaOH |
|---|---|---|---|---|---|---|---|---|
| Cole (1941) | *Homarus* | 26·68 | 1·11 | — | 0·36 | 0·62 | — | — |
| Davenport (1941) | *Cancer* | 28·7 | 0·91 | 1·63 | 1·16 | — | 0·15 | — |
| Hagiwara & Bullock (1957) | *Palinurus* | 26·4 | 1·12 | 2·78 | 0·38* | 0·48† | — | 0·02 |
| Pantin (1934) | *Carcinus* | 31·0 | 0·99 | 1·37 | 2·35 | — | 0·22 | — |
| Prosser (1940) | *Cambarus* | 18·0 | 0·8 | 1·5 | — | — | to pH 7·6 | — |
| Van Harreveld (1936) | *Cambarus* | 12·0 | 0·4 | 1·5 | 0·25 | — | 0·2 | — |

* As $MgCl.6H_2O$.
† As $MgSO_47H_2O$.

byssus retractor muscle is as high as 7·8 (Singh, 1938a). Gatenby (1937a) recommends a saline for marine molluscs which has the advantage of a high calcium concentration but he has omitted to include KCl.

Bozler (1930) was unable to prepare a saline adequate for use with the buccal retractor muscle of *Helix* and used natural blood, a procedure also followed by Ramsay (1940). A useful method of obtaining blood from *Helix* is described by Pantin (1946). The snail is immersed in deoxygenated water for 24 hr allowing osmotic uptake of water to occur. The engorged foot is then sliced and the animal placed in a funnel to drain off the blood. Care should be exercised in the use of whole blood as in some species such as *Viviparus fasciatus* autolysis of organic components results in rapid changes in the osmotic pressure of drawn blood.

The action of various ions on the molluscan heart is discussed by Krijgsman & Divaris (1955) and Robertson (1941) discusses the functions of calcium. As in other animals bicarbonate stimulates the respiration of isolated molluscan tissues (Kerkut & Laverack, 1957). Some confusion has occurred in the literature

AND SALINES FOR CRUSTACEA

given are for amorphous salts

Haemolymph composition—*continued*

| $SO_4$ | pH | Δ°C | Notes |
|---|---|---|---|
| 5·0 | 7·4–7·6 | — | |
| 10–16[3] | 7·4 | — | |
| 2·4 | 7·8[4] | — | |
| — | — | 2·19 | |
| — | — | 0·8[5] | The values given represent the range of analyses. The mean values are in parentheses. |
| — | 7·55[6] | 0·68 | |

Salines—*continued*

| $H_3BO_3$ | pH | $CaSO_4$ | Notes |
|---|---|---|---|
| — | 7·3–7·5 | 3·4 | Buffer with 17·57 ml 0·5 M boric acid plus 0·956 ml 0·5 M NaOH per litre saline. Heart survives up to 26 hr. |
| — | — | — | Plus 1 g/l glucose. For heart. |
| 0·55 | 7·1–7·5 | — | |
| — | 7·0 | — | Plus 60 g/l urea. Muscle survives 8 hr (Pantin, 1934). Satisfactory for heart (Welsh, 1939). |
| — | 7·6 | — | Nerve cord is hyperexcitable in saline with less than 0·8 g/l KCl though this is above the blood K level. CNS survives 2 days. |
| — | 7·5 | — | Heart beats for up to 20 hr (Cole *et al.*, 1939). Unsatisfactory for CNS (Prosser, 1940) but suitable for nerve and neuro-muscular junction. |

[1] Webb (1940). [3] Gilbert (1959). [5] Schwabe (1933).
[2] Shaw (1958). [4] Cole (1941). [6] Van Harreveld (1936).

concerning the saline developed by Barnard & Bonnet (1930). This saline, in which *Helix* heart survives for some 4 days, was prepared by the addition of various salts to a spa water (Evian water); the additives alone in water do not form a satisfactory physiological saline as noted by Goddard (1960). This saline has now been superseded by the better one developed by Julien *et al.* (1955a) and is not therefore listed.

*Gastropods*

Of the terrestrial snails studied the tissues of *Helix pomatia* survive considerably longer in saline than those of *H. nemoralis* or *H. aspersa*. The most satisfactory

composition for salines varies from species to species as does the blood composition. Different species are therefore treated separately. It should be noted that as wide differences are found in the total concentrations of the blood within a single species, and as the analyses quoted are drawn from more than one authority, the values for blood composition should be treated as a rough guide only.

Table 11—The O.P. of the haemolymph of some selected Arthropods

| Genus | Medium | Δ°C Haemolymph | Authority |
|---|---|---|---|
| *Crustacea* | | | |
| *Artemia salina* | 10% sea-water to saturated sea-water | 0·4–1·5 | Croghan (1958) |
| *Cheirocephalus diaphanus* | Fresh-water | 0·43–0·5 | Croghan (1958) |
| *Daphnia* sp. | Fresh-water | 0·24 | von Fritsche (1916) |
| *Ligia oceanica* | Terrestrial | 2·15 | Parry (1953) |
| *Oniscus* sp. | Terrestrial | 1·04 | Parry (1953) |
| *Armadillium vulgare* | Terrestrial | 1·21 | Rouchal (1940) |
| *Porcellio scaber* | Terrestrial | 1·09 | Rouchal (1940) |
| *Asellus aquaticus* | Fresh-water | 0·53 | Lockwood (1959) |
| *Eriocheir sinensis* | Fresh-water | 1·18 | Scholles (1933) |
| *Eriocheir sinensis* | Sea-water | 1·66 | Schlieper (1935) |
| *Potamon* sp. | Fresh-water | 1·17 | Duval (1925) |
| *Palaemonetes varians* | 0·11 Δ°C | 1·05 | Parry (1957) |
| *Palaemonetes antennarius* | Fresh-water | 0·75 | Parry (1957) |
| *Palaemon serratus* | Sea-water | 1·65 | Parry (1954) |
| *Palaemon longirostris* | 0·07 Δ°C | 0·95 | Parry (1957) |
| *Myriapoda* | | | |
| *Iulus* sp. | Terrestrial | 0·42 | Lockwood (Unpubl.) |
| *Arachnida* | | | |
| *Tegenaria atrica* | Terrestrial | 0·78 | Croghan (1959) |
| *Onycophora* | | | |
| *Peripatopsis* sp. | Terrestrial | 0·28–0·54 | Picken (1936) |

Gatenby's saline for marine molluscs has the following composition: NaCl, 23·38; $MgCl_2$, 7·62; $CaCl_2$, 5·55 (g/l).

For the O.P. and ion content of the blood of other gastropods see Saxena (1957), Arvanitaki & Cardot (1931) and Barnard (1931). For the action of ions on *Helix* heart see Wells (1941) and on the gut, Wells (1928). References to other salines used with molluscan hearts are given by Krijgsman & Divaris (1955).

*Lamellibranchs*

*Mytilus* and *Anodonta* are the Lamellibranchs most commonly used in physiological work. Sea-water is used as a saline for *Mytilus*, and Pilgrim (1953), working with the heart, finds that no advantage is gained by raising the potassium concentration of sea-water by 20 per cent to a value nearer that of the blood. On the other hand Potts (1959) finds that the addition of 2 mM/l K and 1 mM/l glucose to sea-water provides the best medium for the maintenance of the ionic composition

of heart muscle though a rapid loss of K does occur from byssus retractor muscle in this medium. Raising the potassium concentration in salines might be expected to diminish the K loss from muscles, and Singh (1938b) has shown that the decrease in excitability of *Mytilus* muscle caused by a high potassium concentration in the bathing medium may be partially counteracted by raising the calcium concentration. Mg should be present in salines used with *Mytilus* as it plays an important part in the maintenance of the intercellular cement in this (Gray, 1926). Frog ringer/5 is a satisfactory medium for *Anodonta* tissues and the isolated heart may beat for up to 2 days in this medium. Somewhat longer survival of the heart occurs in 4% sea-water but the rate of beat is sub-normal (Pilgrim, 1953). A faster heart beat at the expense of longevity is given by a saline based on analysis of the blood.

*Cephalopods*

Despite the fact that much of the present knowledge of nerve physiology has been elucidated by study of squid nerve no special salines have been developed for use with cephalopod tissues. Squid nerve is tolerant of sea-water (Cole & Curtis, 1939), and Hodgkin & Katz (1949) find that there is no difference in the behaviour of nerve in sea-water and their artificial sea-water.

*Insects*

Insects display the greatest range of haemolymph composition found in any group in the animal kingdom. Furthermore, as there are widely different tolerances of changes in the ion content in different species it is difficult to generalize on the behaviour of isolated tissues in saline. For example, a level of over 75 mM/l Mg is required to produce an effect on the muscle action potential in *Carausius* which is given by locust and cockroach muscle in only 10 mM/l (Wood, 1957). Similarly the sensitivity of the neuromuscular junction to the Mg level is also much less in *Carausius* than in the other two forms, yet Mg, except in very low amounts, appears to be toxic to the isolated heart of another herbivorous form, *Samia* (Barsa, 1954). The resting and action potentials of the excitable tissues of most animals are generally sensitive to the K and Na levels in the bathing medium and though this is true of *Carausius* muscle also, an action potential can still be obtained in the complete absence of Na in the medium (Wood, 1957).

A substantial proportion of the total O.P. of the Holometabola is provided by sugars and amino-acids and hence even salines based on ion analyses may prove to be deleterious as a result of their hypotonicity. On *a priori* grounds it would seem to be inadvisable to correct this simply by elevating the NaCl level until the solution is isotonic, as the rate of diffusion of Na into tissues may then in some cases be in excess of the cells' ability to extrude it. Many insect tissues, however, are insensitive to the Na concentration within wide limits and Hoyle (1957) has prepared a satisfactory saline for locust muscle in which the Na concentration is 50 mM/l above the analysed level for the blood.

The sensitivity to the total O.P. of the saline of similar tissues from different species also varies. *Carausius* muscle displays decreased resting and action potentials

in hypotonic saline; but locust muscle does not change its properties though it swells in hypotonic saline. The mitotic activity of grasshopper neuroblasts is greatly influenced by the tonicity of the medium, and the heart rate in *Chortophaga*

TABLE 12—MOLLUSCAN BLOOD

Ions in mM/l,

Blood composition

| Authority | Animal | Na | K |
|---|---|---|---|
| | *Helix pomatia* | 133[1] | 4[1] |
| Huf (1935) | *Lymnaea stagnalis* | 47·5 | 4·26 |
| Pilgrim (1953) | *Anodonta* | 15·5 | 4 |

Salines

| Authority | Animal | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | $NaHCO_3$ | $Na_2HPO_4$ |
|---|---|---|---|---|---|---|---|---|
| Cardot (1921) | *Helix* | 0·6–0·75 Locke solution | | | — | — | — | — |
| Jullien *et al.* (1955a) | *Helix pomatia* | 5·7 | 0·15 | 1·11 | — | — | — | — |
| Kerkut & Laverack (1957) | *Helix pomatia* | 8·6 | 0·21 | 0·34 | 0·13 | — | — | — |
| Jullien *et al.* (1955a) | *Helix aspersa* | 7·6 | 0·335 | 1·0 | — | — | — | — |
| Hedon-Fleig (in Gatenby, 1937) | *Helix aspersa* | 7·0 | 0·3 | 0·1 | — | 0·3 | 1·5 | 0·5 |
| Prosser *et al.* (1950) | *Helix* | 5·87 | 0·73 | 1·99 | 2·7 | — | 1·87 | — |
| Jullien & Ripplinger (1948) | *Lymnaea stagnalis* | 2·7 | 0·3 | 0·83 | — | — | — | — |
| Carriker (1946) | *Lymnaea stagnalis* | 2·0 | — | 0·3 | 0·3 | — | 2·0 | — |
| Pilgrim (1953) | *Anodonta* | 0·936 | 0·019 | 1·0 | 0·035 | — | — | — |
| Potts (1959) | *Anodonta* | 0·819 | 0·037 | 0·555 | — | — | — | — |
| Motley (1934) | *Anodonta* | 1·2 | 0·03 | 0·06 | — | — | — | — |
| Motley (1934) | *Anodonta* | 1·2 | 0·15 | 0·12 | — | — | — | — |
| Hodgkin & Katz (1949) | *Mytilus* | 26·3 | 0·75 | 1·18 | 5·0 | — | 0·22 | — |
| Singh (1938a) | *Mytilus* | 30·0 | 0·76 | 1·16 | — | — | — | — |
| Cambridge *et al.* (1959) | *Mytilus* | 28·3 | 0·74 | 1·21 | 2·6 | 3·53 | — | — |

[1] Jullien *et al.* (1955). [2] Holtz & von Brand (1940). [3] Gatenby *et al.* (1934).

is also influenced by the concentration, being weak and irregular in hypotonic solutions and strong but infrequent in hypertonic media. Barsa (1954) has studied

the action of different ion species on the hearts of *Chortophaga* and *Samia* and concludes in reviewing this and earlier work that insect tissues are unusually tolerant of the cation content of the medium.

COMPOSITION AND SALINES

salts as g/l

Blood composition—*continued*

| Ca | Mg | Cl | $PO_4$ | pH |
|---|---|---|---|---|
| 12[2] | — | 93[2] | 0·23[2] | 8·4[3] |
| 3·05 | 4·8 | 42·6 | — | — |
| 5·3 | 0·36 | 10·5 | — | 7·8–8·08 |

Salines—*continued*

| $KH_2PO_4$ | $KHCO_3$ | $NaH_2PO_4$ | Glucose | pH | Notes |
|---|---|---|---|---|---|
| — | — | — | — | — | Heart survives for 48 hr in 0·7 Locke. Pedal ganglion survives for 24 hr (Hughes & Kerkut, 1956). |
| — | — | — | — | — | Low Ca and K but heart survives 21 days. If glucosamine is added heart survives for 30 days (Jullien *et al.*, 1956b). |
| 0·19 | — | — | — | 7·4 | For tissue respiration. |
| — | — | — | — | — | Saline approximates to blood ionic concentration. Heart survives 24 hr (Jullien *et al.*, 1955a). |
| — | — | — | 1·0 | — | Suitable for respiratory studies on tissues other than the heart (Goddard, 1960). Isolated mantle survives 3 weeks (Gatenby *et al.*, 1934). |
| — | 0·22 | — | — | — | |
| — | — | — | — | — | For maintenance of heart beat. |
| — | 0·1 | — | — | 7·8 | Vas deferens survives for 66 hr as opposed to 12 hr in 0·7% Locke solution (Carriker, 1946). |
| — | — | — | — | 8·1 | For maintenance of heart beat. |
| — | — | 0·035 | — | 7·5 | For maintenance of ionic content of heart muscle. Plus 1 mM glucose and NaOH to pH 7·5. |
| — | — | — | — | 7·5–7·8 | For internal perfuson of heart. |
| — | — | — | — | 7·8 | For external perfusion of heart. |
| — | — | — | As required | — | Anterior byssus retractor remains excitable for 1 to several days. |
| — | — | 0·4 | — | — | After some hours excitability of the byssus retractor is lost as a result of the escape of K from the muscle. |
| — | — | — | 1·0 | 6·2–6·4 | |

Great variation is found in the Na and K concentrations of different species (Duchateau *et al.*, 1953; Boné, 1944) and so, despite the tolerance found by Barsa,

it is anomalous that most of the insect saline at present in use are based on those prepared for vertebrate tissues and few have a Na content of less than 100 mM/l (Hoyle, 1957; Day & Grace, 1959). More satisfactory simple salines could probably be prepared by the technique of Wood (1957) who based the ionic concentration of his saline on blood analyses and then added sucrose until the behaviour of

TABLE 13—BLOOD COMPOSITION AND SALINES

Ions in mM/l,

Blood composition

| Authority | Animal | Na | K | Ca |
|---|---|---|---|---|
| Croghan & Lockwood (1960) | *Drosophila* (larva) | 52 | 36 | — |
| Barsa (1954) | *Chortophaga* | 109 | 3·4 | 2·9 |
| Hoyle (1953) | *Locusta* | 109 | 18 | — |
| Van Asperen & Van Esch (1956) | *Periplaneta* | 156 | 7·7 | 4·2 |

Salines

| Authority | Animal | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $NaHCO_3$ |
|---|---|---|---|---|---|---|
| Villee (1948) | *Drosophila* | 9·0 | 0·4 | 0·2 | — | 0·2 |
| Ephrussi & Beadle (1936) | *Drosophila* | 7·5 | 0·35 | 0·21 | — | — |
| Baumgartner & Payne (1931) | *Chortophaga* | 9·0 | 0·2 | 0·2 | — | 0·2 |
| Barsa (1954) | *Chortophaga* | 13·8 | 0·54 | 0·67 | — | — |
| Gaulden & Carlson (1951) | *Chortophaga* | 6·8 | 0·2 | 0·2 | 0·1 | 0·12 |
| Hoyle (1953) | *Locusta* | 8·2 | — | 0·22 | 0·19 | — |
| Van Asperen & Van Esch (1956) | *Periplaneta* | 9·32 | 0·77 | 0·5 | — | 0·18 |
| Davey (1958) | *Periplaneta* | 10·5 | 1·5 | 2·2 | — | — |
| Griffiths & Tauber (1940) | *Periplaneta* | 14·63 | 0·45 | 0·5 | — | 0·19 |
| Ludwig *et al.* (1957a) | *Periplaneta* | 11·0 | 1·4 | 1·1 | — | — |
| Ludwig *et al.* (1957b) | *Periplaneta* | 9·0 | 0·7 | 0·46 | — | — |
| Pringle (1938) | *Periplaneta* | 9·0 | 0·2 | 0·2 | — | — |
| Taylor (1935) | *Periplaneta* | 9·0 | 0·25 | 0·3 | — | 0·2 |
| Yeager (1939) | *Periplaneta* | 10·93 | 1·57 | 0·83 | 0·17 | — |

muscle was the same as when bathed by haemolymph. Media such as those of Wyatt (1956) which contain high concentrations of K and Mg are unlikely to be successful except for the tissues of holometabolus vegetarians.

It is probable that Mg and Ca and possibly K are not fully ionized in the blood of many insects (Clark, 1958; Barsa, 1954), and Hoyle (1957) has shown that a saline with the measured Mg concentration of locust blood sends muscle into fibrillation. *Chortophaga* heart is also inhibited in similar circumstances. Perhaps when more is known of the ionized level of Mg in the blood, salines may be prepared in which the quantity of this ion is less empirically determined than at present.

Trehalose is frequently present in considerable quantities in insect haemolymph but the addition of this carbohydrate to saline may not give an advantage over the use of glucose in all cases, as Grace (1958) has found that in the absence of endocrine extracts it cannot be utilized by silkworms. On the other hand, Treherne (1960) finds that the isolated nerve cord can rapidly utilize trehalose. A list of some salines

FOR *Drosophila*, LOCUSTS AND *Periplaneta*
salts in g/l
Blood composition—*continued*

| Mg | Cl | pH | Δ°C | Notes |
|---|---|---|---|---|
| — | 30 | 7·1[1] | 0·6 | See Zwicky (1954) for amino acids etc. |
| 21 | — | 6·7[2] | 0·89 | |
| — | — | — | 0·75[3] | |
| 5·4 | 144 | 7·5–8·0[4] | 0·897 | Values for whole blood. Serum levels are given by Tobias (1948). |

Salines—*continued*

| $KHCO_3$ | $NaH_2PO_4$ | $KH_2PO_4$ | Glucose | Notes |
|---|---|---|---|---|
| — | — | — | 2·5 | |
| — | — | — | — | Heart beats for 2 hr. |
| — | — | — | 0·25 | |
| — | — | — | — | Discusses other salines and the effect of ions on the heart. |
| — | 0·2 | — | 7·7 | For neuroblasts. |
| 0·4 | — | 0·82 | — | For nerve cord. |
| — | 0·01 | — | — | |
| — | — | — | — | Sperm live for 5 hr. |
| — | — | — | — | Best of several solutions tested for amplitude of contraction and survival of gut. |
| — | — | — | — | Heart survives 24–36 hr. |
| — | — | — | — | Buffer with Sorenson glycerine buffer to pH 7·5. |
| — | — | — | 4·0 | Nerve cord activity retained for up to 60 hr (Roeder, 1948). |
| — | — | — | — | For haemocytes. |
| — | — | — | — | For maintenance of amplitude and rhythm of heart beat. |

[1] Boche & Buck (1942). [2] Bodine (1926). [3] Rouchal (1940). [4] Roeder (1953).

in use with the tissues of three common experimental insects, *Drosophila*, cockroach and locust, are listed together with the ion concentrations of the haemolymph (Table 13). A number of other insect salines are also given (Table 14). For analyses of the haemolymph of many other insect species see Clark (1958) (Ca and Mg); Boné (1944) (Na and K); Duchateau *et al.* (1953) (Na, K, Ca and Mg); Boche & Buck (1942) (pH); and Rouchal (1940) (O.P.). Buck (1953) has assembled most of the available data from the early literature and gives a list of insect salines. Day & Grace (1959) review the literature on insect tissue culture.

If insect blood is used to bathe isolated tissues, darkening may be delayed by the addition of phenylthiourea (Loeb & Schneiderman, 1956).

TABLE 14—SELECTED SALINES

Values

| Authority | Animal | Tissue | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $NaHCO_3$ | $Na_2HPO_4$ |
|---|---|---|---|---|---|---|---|---|
| Bělar (1929) | *Stenobothrus* | Sperm | 9 | 0·2 | 0·2 | — | 0·2 | — |
| Bishop *et al.* (1925) | *Apis* | Blood cells | — | — | — | — | 0·51 | — |
| Bodenstein (1946) | *Drosophila* | | 7·5 | 0·35 | 0·21 | — | — | — |
| Bradford & Ramsay (1949) | Mosquito | | 7·5 | 0·35 | 0·21 | — | — | — |
| Buck (1953) | *Diptera* | Salivary glands | 5·55 | 0·22 | 0·44 | — | — | — |
| Carlson (1946) | *Chortophaga* | Embryo neuroblast | 7 | 0·2 | 0·2 | 0·1 | 0·005 | — |
| Case (1957) | *Musca* and *Sarcophaga* | | 7·5 | 0·35 | 0·21 | — | — | — |
| Clarke in Gatenby (1937a) | | | 6·5 | 0·14 | 0·12 | — | 0·1 | — |
| D'Angelo (1946) | *Chironomus* | Salivary glands | 3·5 | 6·7 | — | — | — | — |
| Davey (1958) | *Periplaneta* | Sperm | 10·5 | 1·5 | 2·2 | — | — | — |
| Eastham (1925) | *Drosophila* | | 6·0 | — | — | — | — | — |
| | *Calliphora* | | 7·5 | — | — | — | — | — |
| Glaser (1917) | Various Lepidoptera | Blood cells | 9·0 | 0·42 | 0·25 | — | 0·2 | — |
| Hayes (1953) | *Aedes aegypti* | Sperm | 9·0 | 0·2 | 0·2 | — | 0·1 | — |
| Hobson (1928) | *Dytiscus* | Gut muscle | 9·42 | 0·22 | 0·22 | — | 5 drops saturated sol/l | — |
| Hughes (1949) | *Dytiscus* | | 7·84 | 0·75 | 0·40 | — | — | — |
| Levenbook (1950) | *Gastrophilus* | | 1·0 | — | — | — | 0·48 | 11·0 |
| Lévy (1928) | *Phormia* *Calliphora* | Heart | 9·0 | 0·7 | 0·46 | — | 0·16 | — |
| Maluf (1935) | *Anax* *Belostoma* | Heart | 9·0 | 0·2 | 0·2 | — | — | — |
| Millara (1946) | *Carausius* | Haemocytes | 0·38 | 2·0 | 0·8 | — | — | — |
| Takami (1958) | *Bombyx* | Embryos | 7·5 | 0·5 | 0·2§ | — | — | — |
| Wood (1957) | *Carausius* | Muscle | — | 1·34 | 0·832 | 4·75 | — | 1·335 |
| Wyatt (1956) | *Bombyx* | Ovary | — | 2·98 | 0·81 | 3·0† | — | — |
| Wyatt (1956) | *Bombyx* | Ovary | 0·88 | — | 0·11 | 0·16† | — | — |

FOR OTHER INSECTS

in g/l

| $NaH_2PO_4$ | $MgSO_4$ | $K_2HPO_4$ | Glucose | pH | Others | Notes |
|---|---|---|---|---|---|---|
| — | — | — | — | — | — | Many modifications include omitting $NaHCO_3$ and adding 0·01 M phosphate buffer to pH 6·8; and Ca-free Bělar buffered to pH 6·8 with phosphate or bicarbonate for cockroach muscle. |
| — | — | — | — | — | $CaCO_3$ 0·375<br>$MgCO_3$ 0·693<br>$KHCO_3$ 1·43<br>HCl 11·96<br>Lactic acid 0·9 | |
| — | — | — | — | — | — | |
| — | — | — | — | 6·8 | — | M/150 K phosphate buffer to pH 6·8. |
| — | — | — | — | — | — | |
| 0·2 | — | — | 8 | 6·5 | — | Mitotic division occurs for a short time. |
| 0·14 | — | — | — | — | $H_3BO_3$ 1·3 | Perfusion fluid. pH may be adjusted using bicarbonate or phosphate. |
| 0·01 | — | — | — | — | — | |
| — | — | — | — | 7·0 | — | 0·005 M phosphate buffer to pH 7·0. |
| — | — | — | — | — | — | Sperm live for 5 hr. |
| — | — | — | — | — | — | Cytology only. |
| — | — | — | — | — | — | |
| — | — | — | 2·5 | — | — | |
| — | — | — | — | 6·9 | — | Sperm survives 2 hr. |
| — | — | — | — | 7·2 | — | Gut muscle survives for "many hours". |
| — | — | — | 5·0 | — | — | Gives better results than Pringle's saline. |
| 5·3* | 4·0‡ | 1·5 | — | 6·65 | — | Make up without bicarbonate or $MgSO_4$ as p.p.t. of magnesium polyphosphate occurs on standing. |
| 0·01 | — | — | — | — | — | Best of salines tried for maintenance of heart beat. |
| — | — | — | — | — | — | Heart beat for 2 hr. |
| — | 2·0 | 0·9 | 20·0 | — | — | |
| 0·05* | — | — | 2 | — | Water 750 | To 750 ml solution add 250 ml water containing 0·05 g $NaHCO_3$. |
| 0·21 | — | — | 63·3 | 6·6 | — | |
| 1·1 | 3·7 | — | 0·7 | — | Sucrose 0·4<br>Fructose 0·4 | Should be used only with vegetarian holometabolus insects. With added amino-acids ovary of *Bombyx* survives 3 weeks. |
| — | — | — | — | — | — | If mixed with 10% haemolymph this solution gives survival of ovary for 5–6 days. |

* As $NaH_2PO_4 2H_2O$. † As $MgCl_2 6H_2O$. ‡ As $MgSO_4 7H_2O$. § As $CaCl_2 2H_2O$.

More complex media including glutamine, glycine and other amino- and organic acids have been used by Wyatt (1956), Ramsay (1954), Shaw (1956), Loeb & Schneiderman (1956) and Jones & Cunningham (1960).

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## 4. BASIC REQUIREMENTS OF SALINES AND NOTES ON THEIR PREPARATION

It is difficult to make generalizations with regard to the best method of preparing a physiological saline since biological processes are maintained in a dynamic state of "normality", and the optimum concentration of the various ions in a saline depends both on the relative sensitivity of the homeostatic mechanisms to the ion species and on the specialized function of the particular tissue in use. A single physiological saline is unlikely to provide optimum conditions for all the tissues of an animal and the ion composition must be adjusted according to the tissue and the tissue function under study. Two examples will suffice to illustrate this point. A high Ca concentration in the medium may have an adverse effect on the excitability of nerves and muscles but may be beneficial to other tissues by restricting the rates of diffusion of ions and water, and hence the active work to be performed in extruding Na. A high K concentration in the saline may decrease the tendency for K loss from isolated tissues but will diminish the resting potential of nerves and muscles.

Obviously the best criterion of success for a saline is that an isolated tissue should behave in it as it does in the body of the intact animal. Whilst this can never be fully achieved in fact, the procedure adopted by Wood (1957) when preparing a saline for *Carausius* muscle illustrates a satisfactory approach to the study of a particular system. Wood determined the resting potential of intact muscle fibres bathed by haemolymph, made up a saline in which the ion levels conformed to his analyses of the haemolymph composition and then empirically added sucrose until the potential of isolated muscles fibres bathed by the saline was the same as in the intact muscle. Whilst salines should be based, as in this case, on the ionic composition of the haemolymph, care must be taken in selecting values quoted in the literature for the concentration of K, Ca and Mg. Such values, particularly in the case of invertebrates and lower vertebrates, are often for whole blood and thus include haemocyte K. The K level given may thus be in excess of the true level of ionized K in the blood. Recorded Mg and Ca levels are also usually in excess of the ionized levels as these ions are in part bound to the organic constituents of the haemolymph.

Most tissues tend to lose K and gain NaCl and water when first isolated and placed in physiological saline. In some cases this tendency may be reversed later and the tissue partially regain the K and extrude the NaCl and water if a respiratory substrate is present. This recovery can be assisted further by raising the external K and Ca levels of the saline. Such a procedure may, however, affect the electrical properties of excitable tissues, and the alternative method of raising the $HCO_3$ level to about 30 mM/l (Harris, 1953; Creese, 1952) seems therefore to be preferable at least for muscle.

Glucose and sucrose are frequently added to salines both as respiratory substrates and to make hypotonic solutions up to isotonicity with body fluids. The effectiveness of the addition of respiratory substrates in improving or maintaining the metabolic activity of an isolated tissue depends both on the degree of ionic

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activity at the cell surface and on the degree to which metabolites are stored in the tissue. Thus, as Ussing (1960) points out, brain and kidney tissues combine a high turn-over of ions with a limited internal energy store whilst muscle usually has an adequate reserve of glycogen. In consequence rather little response may be expected from the addition of a respiratory substrate in short-term experiments with muscles. This seems to be supported by the failure of Harris (1953) to find a respiratory substrate which would improve on the retention of K by muscles in bicarbonate saline. Brain slices on the other hand respond to a number of tricarboxylic cycle acids (Ussing, 1960), whilst a mixture of 20 mM/l glucose and 10 mM/l glutamate is also effective in improving the K retention (Terner *et al.*, 1950). A-ketoglutarate is a suitable substrate for kidney slices (Krebs *et al.*, 1951) whilst glucosamine is particularly effective in maintaining the heart beat of *Helix* (Julien *et al.*, 1955b).

Many invertebrates and particularly insects and nematodes have a high level of trehalose in the haemolymph (Fairbairn, 1958), but the use of trehalose has not been found to give any advantage over glucose in salines for the silkworm (Grace, 1958). However, as Treherne (1960) finds that this sugar is rapidly utilized by the nerve cord of the cockroach, its use with insect and other invertebrate tissues seems to be justified pending further clarification of the ability of different species to metabolize it.

Adequate buffering must be present to last for the duration of the experimental period, but it should be stressed that whilst both $HCO_3$ and $HPO_4$ are necessary for some metabolic activities high concentrations of the latter may exceed the solubility product of calcium phosphate and consequently precipitate Ca from the medium. The same consideration limits the general use of citrates and succinates as buffers. Ca may also be precipitated from salines containing $HCO_3$ if they are allowed to lose $CO_2$. Bicarbonate salines should therefore not be sterilized and, if stored, should be in glass-stoppered bottles. Ideally stock solutions should be stored in a refrigerator and phosphates and respiratory substrates should be added only immediately prior to use in order to avoid the acceleration of bacterial growth. Salines stored at low temperatures must be brought up to the temperature of the tissues before use or the cellular ion balance may be disturbed. As an additiona' precaution many authors recommend that each salt should be made up and stored separately, mixing being carried out only immediately prior to use.

Solutions in which tissues are bathed should be aerated but not gassed with mixtures containing more than 3–5% $CO_2$ as the active transport of sodium is inhibited by 5–8% $CO_2$.

## SUMMARY

A saline should be reasonably effective in temporally maintaining the tissues of an animal if it has the following basic features: Modifications may be made as necessary for particular functions.

(1) Na as in the blood.

(2) Cl as in the blood.

(3) K and Ca as in the blood, or somewhat raised.
(4) Mg at blood level or somewhat reduced to allow for organic chelation.
(5) Adequate buffering at the correct pH preferably with both $HCO_3$ and $HPO_4$ present.
(6) Respiratory substrate to make saline up to isotonicity with the haemolymph.
(7) Adequate aeration.
(8) Appropriate temperature.
(9) For long-term experiments the solution should be changed at frequent intervals, or should be allowed to flow over the tissue. If unchanged, 30 mg/l sodium penicillin is sometimes a useful additive.

If salines are to be prepared for animals whose blood has not been analysed it should be noted that the ratio Na + K : Ca + Mg is in the range 10–25 for vertebrates but only 5–15 for invertebrates.

Further information on the preparation of salines is given by Cameron (1935), Strangeways (1924), Parker (1950), Willmer (1954) and Day & Grace (1959).

Culture methods for a considerable variety of invertebrates are described by Galtsoff *et al.* (1937).



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# SOME PHYSIOLOGICAL ASPECTS OF GLUTAMATE–ASPARTATE TRANSAMINATION IN INSECTS

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**Abstract**—1. The quantitative distribution of glutamate–aspartate transaminase in several tissues of the roach was demonstrated and the enzymic activity relative to both protein content and tissue weight is presented. Significant differences between tissues were observed.

2. Centrifugal fractionation of roach muscles showed that transaminase activity is mainly localized in the soluble fraction of the sarcoplasm.

3. Glutamate–aspartate transaminase activity was followed throughout the life cycle of the housefly. During larval growth the concentration and specific activity of the enzyme increased, then fell during the pupal stage. Enzymic activity rose sharply during adult emergence. Similar quantitative determinations were made using several tissues during adult development of the cecropia silkworm. The only striking increase in transaminase activity during adult development occurred in the thoracic muscles.

4. It is suggested that the U-shaped curve of transamination is related to U-shaped curve for protein synthesis during morphogenesis and that the main site for the interconversion of amino acids during adult development may be the muscles.

## INTRODUCTION

THE life cycle of the higher orders of insects is characterized by an extreme change in form, termed metamorphosis, which is accompanied in many species by a temporary interruption in the course of developmental events. This interruption, diapause, is observed as a cessation of cellular growth and as a period of very low cellular respiration. The pupa has been described as a cleidoic system. Its metabolism depends upon food reserves accumulated during the preceding period of high activity, the larval stage. The formation of the adult is the result of a reorganization of the pupal reserves into metabolites which can be used in the synthesis of adult tissues. The latent developmental machinery of the diapausing pupa is triggered by certain endocrinological events, so aptly reviewed by Williams (1958).

It is obvious that the hormonal activity is impressed on, and expressed by, metabolic events in the cells. Although great strides have been made in the elucidation of the hormonal basis of growth and metamorphosis in insects, the

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metabolic aspects of these processes have lagged behind. This does not mean that little is known of the biochemical description of metamorphosis and especially diapause; actually a wealth of information exists (Needham, 1950; Wyatt, 1958). It is difficult, however, to unravel *in vivo* the causal relationship between hormonal action and the metabolic events in differentiation.

With the advent of crystalline insect hormones, attempts have been made to find the site of primary action of these hormones. Wigglesworth (1957) feels that "the essential defect in the dormant tissues of *Rhodnius* seems clearly to be a failure of protein synthesis". Upon the administration of ecdysone the ability to synthesize proteins in this insect is restored within hours. Because it is generally accepted that the building blocks for protein synthesis come from the amino acid pool maintained mainly by transamination (Meister, 1955), it was thought that a study of transamination in insects during growth and metamorphosis might provide additional information on the biochemical events of growth and metamorphosis.

The various parameters affecting the activity of glutamate–aspartate transaminase have been studied in another communication (McAllan & Chefurka, 1961). Within the framework of this information, we now explore such problems as the distribution of this enzyme in insect tissues, its intracellular location, and its activity during morphogenesis.

## MATERIALS AND GENERAL METHODS

### *Insect material*

The insects examined in this study were taken from standard laboratory cultures maintained under constant conditions. All roaches (*Periplaneta americana*) tested were adults, and no attempt was made to select for sex. The ages of all stages of houseflies (*Musca domestica*) were known within 3 hr, and within 1 hr in the case of eggs. The stage of adult development of *Hyalophora cecropia* was determined using a key to morphological characteristics prepared by Schneiderman & Williams (1953).

Tissues of the roach and cecropia silkworm were dissected in chilled Ringer's solution, the wet weight measured, and homogenized at 700 rev/min for 3 min in water in a Potter–Elvehjem homogenizer immersed in an ice bath. Whole specimens of the various stages of the housefly were treated similarly.

### *Transaminase assay*

Glutamate–aspartate transaminase activity was assayed by recording the change in optical density at 280 m$\mu$ due to the formation of oxaloacetic acid. The reaction mixture contained 0·5 ml of 0·1 M aspartate, 0·5 ml of 0·1 M $\alpha$-ketoglutarate, 0·1 ml of homogenate and 3·9 ml of borate buffer, pH 8·3.

### *Protein determination*

The concentration of protein in the homogenates was determined after the method of Lowry *et al.* (1951) using phenol reagent, with crystalline serum albumin as standard.

## RESULTS

### *The distribution of glutamate–aspartate transaminase in the tissues of the roach*

Kilby & Neville (1957) have shown that both glutamate–aspartate and glutamate–alanine transaminases are present in the fat body, Malpighian tubules, and midgut of *Schistocerca gregaria*, and Barron & Tahmisian (1948) have shown glutamate-alanine transamination in roach flight muscle. Several authors (Bheemeswar & Sreenivasaya, 1952; Fukuda, 1957; Shyamala & Bhat, 1955) have made interesting

Table 1—The glutamate–aspartate activity* of the tissues of the roach

| Tissue | Specific activity† | Units per mg tissue |
|---|---|---|
| Malpighian tubules | 1·6 ± 0·25 | 0·18 ± 0·013 |
| Fat body | 0·5 ± 0·22 | 0·16 ± 0·012 |
| Nerve cord | 1·3 ± 0·25 | 0·029 ± 0·001 |
| Muscle (thoracic) | 1·1 ± 0·35 | 0·057 ± 0·014 |
| Foregut | 1·1 ± 0·56 | 0·064 ± 0·009 |
| Midgut | 0·7 ± 0·13 | 0·045 ± 0·004 |
| Hindgut | 1·0 ± 0·20 | 0·051 ± 0·005 |

* Units are defined as that enzymic activity producing 0·588 mg of oxaloacetate in 10 min at 22°C in the standard reaction containing 0·5 ml of 0·1 M aspartate, 0·5 ml of 0·1 M α-ketoglutarate, 3·9 ml of borate buffer, pH 8·3, and 0·1 ml of homogenate. Concentration of oxaloacetate was determined at 280 mμ using the millimolar extinction coefficient 2·92.

† Specific activity is the units of enzymic activity per milligram of protein.

observations on transamination in the silkworm. Most of the reported experiments were of a semi-quantitative nature. It was considered, therefore, that the quantitative determination of glutamate–aspartate transaminase in various tissues of the roach might indicate a possible physiological importance of this enzyme in the interconversions of amino acids in the tissues.

Statistical analysis, using the *t* test, of the data given in Table 1 showed that, on the basis of weight of tissue, enzymic activity in Malpighian tubules and fat body was significantly greater (at the 1 per cent level) than that for other tissues, and that activity in nervous tissue was significantly (at the 5 per cent level) less than the activity in other tissues. However, the specific activities in Malpighian tubules and nerve cord were significantly (at the 10 per cent level) greater than that in fat body. No change in optical density of the glutamate–aspartate reaction mixture was observed in tests using roach blood even after 60 min incubation; therefore, it was concluded that glutamate–aspartate transaminase is not present in roach haemolymph in measurable quantity.

### *The intracellular distribution of glutamate–aspartate transaminase in the roach thoracic muscle*

Continuing the determination of the loci of glutamate–aspartate transaminase activity, the cellular fractions of roach thoracic muscle were separated and the

specific enzymic activity of these fractions measured. The procedure described by Chefurka (1957) was used. Flight muscles were dissected out in cold Ringer's solution, and 300 mg of tissue ground with a Potter–Elvehjem homogenizer in cold sucrose or sucrose and versene. Two concentrations of sucrose, 0·25 M and 0·36 M, with and without versene, were used as media to find the medium which best protected the muscle mitochondria and other particles during fractionation. After

TABLE 2—INTRACELLULAR DISTRIBUTION OF GLUTAMATE–ASPARTATE TRANSAMINASE IN ROACH THORACIC MUSCLES

| Fraction | Specific activity* | Units of total activity | % recovery |
|---|---|---|---|
| 0·25 M sucrose | | | |
| Homogenate | 0·43 | 4·55 | (100) |
| Mitochondria | 0·08 | 0·48 | 9·5 |
| "Microsomes" | 0·60 | 0·11 | 2·4 |
| Supernatant | 0·95 | 2·13 | 47·0 |
| 0·25 M sucrose + 0·01 M versene | | | |
| Homogenate | 0·28 | 4·22 | (100) |
| Mitochondria | 0·03 | 0·39 | 9·5 |
| "Microsomes" | 0·50 | 0·14 | 3·6 |
| Supernatant | 0·94 | 2·63 | 63·0 |
| 0·36 M sucrose + 0·01 M versene | | | |
| Homogenate | 0·20 | 2·94 | (100) |
| Mitochondria | 0·02 | 0·26 | 9·0 |
| "Microsomes" | 0·40 | 0·27 | 9·0 |
| Supernatant | 0·51 | 2·15 | 73·0 |
| 0·36 M sucrose | | | |
| Homogenate | 0·20 | 3·12 | (100) |
| Mitochondria | 0·02 | 0·22 | 7·0 |
| "Microsomes" | 0·46 | 0·32 | 10·0 |
| Supernatant | 0·63 | 1·96 | 63·0 |

* Specific activity is units of enzymic activity per milligram of protein (see Table 1).

grinding, the extracts were filtered through eight layers of muslin moistened in the medium used, and the residue washed with the isolation medium. The residue consisted mainly of muscle fibres. The filtrate and washings were collected in a volumetric cylinder and 0·5 ml taken to determine transaminase activity and protein content. The filtrate was centrifuged at 21,000 *g* for 10 min in a Spinco (Model L) ultracentrifuge, the supernatant decanted, and the mitochondrial pellet was suspended in 2 ml of medium and re-centrifuged. The resuspension and centrifugation of the mitochondrial pellet were repeated. The final pellet was suspended in 4 ml of medium and assayed for transaminase activity and protein content. Although the final pellet may contain some nuclei, it was predominantly composed of muscle mitochondria. A portion of the combined supernatants was then sampled for assay while the remainder was centrifuged at 57,000 *g* for 1 hr. While the nature of this second residue was not identified it will be termed "microsomal" because it is within these gravitational forces that microsomes are isolated from mammalian

tissues. The supernatant was decanted, and the resulting pellet was resuspended in 3·0 ml of medium. Both fractions were sampled for transaminase activity and protein content.

Most of the enzymic activity was recovered in the non-particulate supernatant (Table 2). Although the average recovery of total activity using 0·25 M sucrose was only 56 per cent, the values of the higher total recovery using 0·35 M sucrose strengthened the conclusion that glutamate–aspartate transaminase is located in the sarcoplasm of roach thoracic muscles. It is also noteworthy that the recoveries of total activity were improved by the addition of versene, and that it was the recovery of the activity in the supernatant fraction that was predominantly increased by this chelating agent. It is possible that the enzymic activity observed in the particulate fractions was due to adsorption.

*Glutamate–aspartate transaminase activity throughout the life cycle of the housefly,* Musca domestica

The transformation of holometabolous insects during the pupal stage from the larval to the adult form involves biochemical events which manifest themselves in

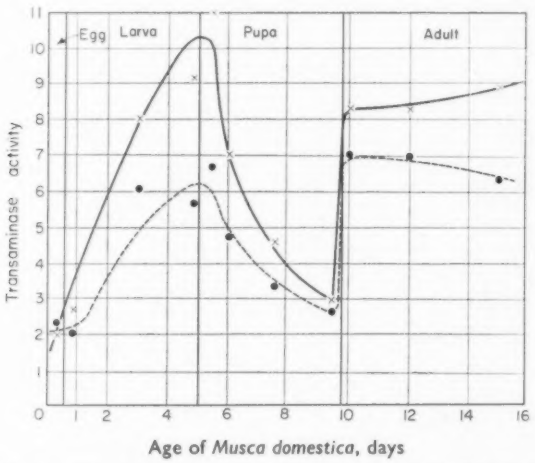


Fig. 1. Change in glutamate–aspartate enzymic activity during the life cycle of the housefly. Transaminase activity expressed as units of enzyme per milligram of tissue (X——X) and units per milligram of protein (● – – ●). All points are the means of three determinations.

the dissolution of some larval tissues and the appearance of other adult tissues. Such a morphological transformation would presumably involve considerable protein metabolism. Protein synthesis requires the maintenance of an amino acid pool as a source of residues. Since transamination is the main regulatory mechanism of the amino acid pool, a study of transamination during the metamorphosis of insects should be of interest. Therefore the glutamate–aspartate activity of the housefly during its life cycle was investigated.

Samples of the various stages of the housefly were examined for enzymic activity and protein content, as already described. The results presented in Fig. 1 emphasize the fact that both the enzymic activity per milligram of tissue and the specific activities follow a U-shaped pattern. The changes in enzymic activity are statistically significant. Furthermore, the protein concentration remains constant throughout the larval, pupal and adult stages. Therefore, the changes in specific transaminase activity are not associated with changes in the protein levels during morphogenesis.

*Transaminase activity during adult development of* Hyalophora (Platysamia) cecropia

In the light of the relationship between morphogenesis and transamination revealed above, it was considered necessary to follow the transaminase activity in various tissues of an insect during adult development, to determine whether the changes in transaminase activity were general, or whether the rate of change varied



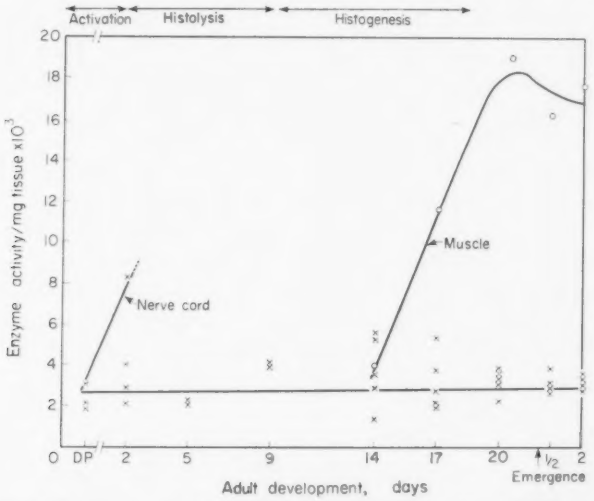


FIG. 2. Change in glutamate–aspartate enzymic activity of the tissues of *H. cecropia* during adult development. The horizontal line represents the mean of the activities of blood, midgut, Malpighian tubules, fat body and nerve cord except as shown. Assay of enzymic activity was begun in diapausing pupae (DP). Adult development was reckoned from the day the pupae were placed at 25°C after chilling at 5°C (Schneiderman & Williams, 1953). Histolysis of tissues precluded testing of certain tissues on the fifth and ninth days of adult development.

in different tissues. For these experiments *Hyalophora cecropia* was selected. Fifty female pupae were stored at 5°C. When removed to 25°C adult development began immediately.

Several stages were selected during development, since insufficient material was available for a more complete survey. These stages were: diapausing pupae

taken from the stock at 5°C, samples from the second, fifth, ninth, fourteenth, seventeenth and twentieth day stages, adults 6–12 hr after emergence, and 2-day old adults. Insects in these nine stages were dissected under cold Ringer's solution, and the tissues weighed and homogenized in a known volume of water. Homogenates were assayed for protein content and transaminase activity.

Table 3—Protein content and transaminase activity in developing cecropia muscle

| | Development stage | | | | |
|---|---|---|---|---|---|
| | Fourteenth day | Seventeenth day | Twentieth day | Half-day adult | Two-day adult |
| Protein content (mg protein/mg tissue) | 0·124 ± 0·007 | 0·076 ± 0·004 | 0·062 ± 0·004 | 0·096 ± 0·004 | 0·122 ± 0·005 |
| Enzymic activity (units/mg tissue) | 0·015 ± 0·002 | 0·035 ± 0·002 | 0·055 ± 0·008 | 0·047 ± 0·008 | 0·041 ± 0·006 |

Of the tissues assayed only thoracic muscles showed a marked increase from a very low level of enzymic activity to values comparable with those of most roach tissues and adult flies (Fig. 2). Haemolymph, fat body, Malpighian tubules and the three divisions of the gut possessed low transaminase activity in all stages. The persistence of this low activity in most tissues is puzzling, especially in view of the high enzymic activity of corresponding tissues in the roach, and the increase from low to high rate of transamination in the development of the adult housefly. During early adult development, the nerve cord showed an increase in activity, but as development progressed the cord could not be extirpated with ease. In later development when the nerve cord could again be easily removed it showed the general low activity. Since thoracic muscle alone showed a marked increase in transaminase activity to a level comparable with that in adult flies, it may be that the increase in transamination during metamorphosis of the housefly was associated with this specific tissue.

It is conceivable that the transaminase activity in developing flight muscles of *H. cecropia* functions to regulate the constitution of an amino acid pool which provides the residues for protein synthesis, particularly as this synthesis may be extensive during muscle formation. Certainly marked changes in the gross appearance of this tissue take place from the fourteenth to the twentieth days. The change in protein content of muscle during development is compared with transaminase activity in Table 3. Protein concentration decreased as transaminase activity increased up to the twentieth day of development, then the protein concentration increased while the transaminase activity remained constant.

## DISCUSSION

Generally, any attempts to establish a clear-cut relationship between transamination and protein synthesis have yielded inconclusive results (Braunstein,

1947). It is suggested, however, that a "balanced amino acid pool" is necessary for protein synthesis and that transamination is the main general mechanisn for the regulation of this pool (Meister, 1957). The data presented in this paper indicate a close association between high transaminase activity and growth and development in two species of insects. It is probable that as new tissues are formed from the primordial elements, the histoblasts, transamination serves to regulate the supply of building blocks for protein synthesis in these tissues during growth.

One of the characteristic biochemical features of insect growth and development is the U-shaped curve of respiration. Generally, this is closely associated with the total quantity of respiratory enzymes (Wolsky, 1938; Schwann, 1940; Agrell, 1949; Bodenstein & Sacktor, 1952; Schneiderman & Williams, 1954; Shappirio & Williams, 1957). More recently it has been suggested that in the main this U-shaped curve in *Rhodnius* is a curve of protein synthesis (Zwicky & Wigglesworth, 1956). These authors found that during the upswing of the U-shaped respiration curve, there is an increase in cytoplasm, in cell number, formation of chitin and protein in the cuticle. The data in this paper show that transaminase activity during insect growth and development can also be described by a U-shaped curve. The increase in transaminase activity during the upswing of the U-shaped respiration curve suggests a close relationship between protein synthesis and transamination. The fact that in the cecropia silkworm this U-shaped activity of transamination is confined to the developing muscles suggests that muscles may be the centre of transamination during development. The muscles may maintain a pool of amino acids for protein synthesis in other tissues.

Glutamate–aspartate transaminase appears to be unequally distributed in the various organs of the roach. The high enzymic activity of the Malpighian tubules may be associated via glutamic dehydrogenase with their intense carbohydrate metabolism (Chefurka, unpublished) which in turn may provide the necessary energy for their main function—excretion. The transaminase activity per milligram of fat body coupled with the size of this organ suggests that it too may act as a major site of amino acid interconversion in the roach, thus maintaining the high amino acid level in the roach haemolymph.

The data reported in this paper also provide a beginning into an understanding of the sites of transamination within the cells of insect tissues. If the muscle is indeed the site of production of building blocks for protein synthesis during morphogenesis, it appears that interconversion of the amino acids occurs within the sarcoplasm. It is difficult to assess the generality of this conclusion mainly because very little is known of the intracellular localization of transaminase in mammals (Schneider, 1959) and because studies on the fat body of locusts have indicated that an appreciable activity is found in the mitochondrial fraction (Kilby & Neville, 1957). Whether this difference in intracellular localization of a transaminase in muscle and fat body is a function of the difference in the composition of the isolation medium or a true difference between tissues must await further work. Some variation between tissues in intracellular distribution of enzymes

might be expected. Such differences have already been noted with regard to the localization of hexokinase in the mitochondrial fraction of brain (Crane & Sols, 1953) and the cytoplasmic fraction of liver (Hers *et al.*, 1951).

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# BOOK REVIEW

**Polypeptides which Affect Smooth Muscle and Blood Vessels.** Edited by M. SCHACHTER. Pergamon Press, Oxford, 1960. vi+336 pp., 50s.

THIS book contains the proceedings of a symposium held in London in March 1959. It was the fourth in the Biological Council's Coordinating Committee for Symposia on Drug Action, under the chairmanship of Sir Charles Harington. In his opening remarks the chairman stated that the symposium dealt with polypeptides, the majority of whose molecular weights fell in the range 1000–2000. The latter part of the symposium, however, is concerned with higher molecular weight polypeptides, for example leucotaxine and exudin. Antibiotic peptides are not discussed in this symposium.

The last symposium was held in Montreal 1953 and the results published as "Polypeptides which Affect Smooth Muscle" (Edited by Gaddum; Livingstone). Since then research interest in this topic has increased and this is reflected in the present volume. The book has been divided into sections. *Section* I on Oxytocin and Vasopressin contains four papers on the inactivations of these peptides by chemical methods and by enzymes (K. C. Hooper; H. Tuppy; S. E. Dicker; H. Heller). Oxytocinase and vasopressinase are described as separate enzymes, and the removal of the peptides by the kidneys is also described. Homologues and analogues of oxytocin and vasopressin have been prepared and Konzett describes the assay of these together with the actual peptides. Other papers on these two peptides were on the clinical application of oxytocin (G. W. Theobald); measurements of these peptides in body fluids (J. M. Walker) and the control of release (M. Pickford).

*Section* II. Recently Angiotensin was purified and its amino acid sequence determined independently by Skeggs, and by Peart and Elliott. It was first synthesized by Schwzyer. In the section dealing with Angiotensin, papers are presented by these leading contributors.

Substance P is the subject of *Section* III. Its distribution is discussed (J. H. Gaddum) as also its effect on smooth muscle (B. Pernow); its role in the nervous system (G. Zetler) and role in degenerating nerve (P. Holton).

*Section* IV covers Kallidin, Bradykinin and related substances. Wasp venom contains a kinin that is a muscle stimulant and a hypotensive agent (M. Schachter). Other papers by M. Forell, E. W. Horton, C. Keele, S. M. Hilton and M. Roche e Silva discuss various aspects of these kinins. Elliott in the discussion reported that bradykinin had its amino acid sequence determined.

The final section on higher molecular weight peptides included Anaphylatoxin (F. Hahn); Leucotaxine and Exudin in relation to inflammation (V. Menkin), the latter two compounds being a polypeptide and a mucoprotein respectively.

There is no doubt that such a volume will do much to summarize and concentrate the knowledge in this rapidly growing field. It is perhaps to be regretted that the discussions were often short, but nevertheless much of the interest is conveyed. This volume together with the recent review by G. P. Lewis (*Physiol. Rev.* 1960, **40**, 647–676) help to provide the reader with an up-to-date account and a very useful survey of the polypeptides of biological importance.

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